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(54) Title: ENZYME

(57) Abstract: A thermostable xylanase enzyme capable of modifying a xylan polymer in a food and/or feed supplement. The activity of the xylanase enzyme is substantially independent of any level of a wheat xylanase inhibitor that may be present in the food and/or feed supplement.

ENZYME

FIELD OF THE INVENTION

The present invention relates to an enzyme. The present invention also relates to a nucleotide sequence encoding same.

In particular, the present invention relates to nucleotide sequence encoding a xylanase enzyme.

The present invention also relates to the use of the enzyme and nucleotide sequences encoding same in the modification of a food and/or feed supplements comprising a xylan polymer.

BACKGROUND OF THE INVENTION

Plant biomass comprises on average 23% lignin, 40% cellulose and 33% hemicellulose by dry weight (Coughlan & Hazlewood, 1993). Hemicellulose, one of the most abundant of organic substances in existence, accounts for 5-50% of the dry weight of plant materials (Dekker & Lindner, 1979). Xylans constitute the main polymeric component of hemicellulose, being present in the cell walls of all land plants, particularly in tissues that have undergone secondary thickening, where they are known to have important structural functions (Horton & Wolfrom, 1963), Xylans are also present to some extent in the primary walls of growing cells, seeds and bulbs of certain plant species, functioning as reserve polysaccharides.

Structurally, xylans are complex heteropolysaccharides based on a backbone structure of β-1,4-linked D-xylanopyranose units and, depending on their source and method of extraction, they may be substituted and may be linear or branched (reviewed in Puls & Poutanen, 1989). Substitutions include: acetylation at C-2 or C-3 of the xylose units; α-1,2 linked 4-0-methyl- glucuronic acid groups; α-1,3-linked arabinofuranose units; and ferulic or coumaric acids esterified to C-5 of arabinose (Puls & Poutanen, 1989).

By way of example, the xylans of hardwoods, softwoods and grasses are, on average, comprised of 70-200 D-xylopyranose units joined by β-1,4-linkages and, depending on their source and method of extraction, they may be substituted and may be linear or branched (Puls & Poutanen, 1989). Very few unsubstituted linear xylans have been isolated, the best known being from the xylans from esparto grass, tobacco stalks and guar seed husk.

Xylans having a β-1,3-linked backbone are found only in marine algae (Dekker & Richards, 1976), where, in certain species, they form highly crystalline fibrillar structures in the absence of cellulose. Mixed link β-1,3- and 1,4-xylans are found in certain seaweeds such as *Palmaria palmata* (formerly *Rhodymenia palmata*) (Barry & Dillon, 1940). Hardwood xylan (10-35% of dry weight) is typically 0-acetyl-4-0-methylglucuronoxylan (Degree of Polymerisation (DP) 200). Approximately 10% of the backbone xylose units are α-1,2-linked to a 4-0-methyl-a-D-glucuronic acid residue, while 70% of the xylose residues are acetylated at C-2 or C3, or both. Most hardwood xylans contain small amounts of rhamnose and galacturonic acid, as integral components of the main chain.

The xylan of softwoods (10-15% of dry weight) is an arabino-4-0-methylglucuronoxylan (DP >120) and differs from that of hardwoods in not being acetylated. Substituents are 4-0-methylglucuronosyl residues attached to C-2 and L-arabinofuranosyl residues attached to C-3 of the relevant xylose backbone units. The average ratio of the sugar units is 100:20:13 (Xyl:4-0-Me-GlcA:Ara) (Puls & Poutanen, 1989). The xylan of grasses and cereals is also an arabino-4-0-methylglucuronoxylan (DP 70), but it has a lower 4-0-methylglucuronic acid content than does hardwood xylan, and a larger proportion of L-arabino-furanosyl side-chains, linked to C-2 or C-3, or both of the xylose main chain residues (Voragen *et al.*, 1992). These xylans also contain 2-5% by weight of 0-acetyl groups linked to C-2 or C-3 of the xylose units (Bacon *et al.*, 1975). Approximately 6% of the arabinosyl side-chain residues are substituted at C-5 with feruloyl groups, while approximately 3% are substituted at C-5 with p-coumaroyl residues (Mueller-Harvey *et al.*, 1986). The relative proportions of the substituents vary from species to species. Feruloyl and coumaroyl groups have been

implicated in the formation of covalent linkages between xylans, xylan and lignin, and between xylan and other cell wall polysaccharides, particularly galacturonans (Joseleau *et al.*, 1992). It is not surprising, therefore, that the structural complexity of xylans is reflected in the consortium of main- and and side-chain-cleaving enzymes that act cooperatively to effect complete hydrolysis.

In this regard, the extensive breakdown of xylans requires the synergistic action of a variety of hydrolytic enzymes, the xylan-degrading enzyme system (Biely *et al* 1985, Puls & Poutanen, 1989). Depending on the origin of the cell wall material such enzymes include the main chain-cleaving enzymes, the endo-1,4-β-xylanases (EC 3.2.1.8) and the β-xylosidases (EC 3.2.1.37) those that liberate side chain substituents, namely α-glucuronidases, acetylxylanesterases and α-L-arabinofuranosidases (EC 3.2.1.55).

The main-chain enzymes involved are endo-β-1,4-xylanase (β-1,4-D-xylan xylanohydrolase: EC 3.2.1.8), β-1,4-xylosidases (β-1,4-D-xyloside xylohydrolase: EC 3.2.1.37) and possibly exo-xylanases (β-1,4-D-xylan xylohydrolase). Depending on the source of the xylan, side-chain cleaving activities necessary include α-glucuronidase and acetylxylanesterase (EC 3.1.1.6), as well as the esterases that liberate acetyl, coumaroyl and feruloyl substituents. Many of these enzymes have been shown to interact synergistically, e.g. the removal of side-chain substituents to facilitate hydrolysis of the backbone by endoxylanse (Lee & Forsberg, 1987). Conversely, several accessory enzymes will only remove side-chain groups from xylo-oligosaccharides, generated by the prior action of xylanase. The various types of synergistic interactions may be distinguished and described as homeosynergy, heterosynergy, uni- and bi-product synergy and antisynergy (Coughlan *et al.*, 1993).

Endoxylanase, as the name implies, cleaves β-1,4 linkages in the backbone of xylans, arabinoxylans, glucuronoxylans and related polymers (Coughlan *et al* 1992). However, depending on the enzyme, such action may require, or be hindered by, side chain substitution of the substrate (for a review see Coughlan *et al* 1992). β-xylosidases cleave xylobiose, remove xylose residues from the non-reducing ends of xylo-oligosaccharides, and many can also liberate xylose from artificial substrates such as

aryl β-xylosidases. Generally, β-xylosidases do not act against 'native' xylans although exceptions have been reported. An example of the latter is the enzyme from *Neocallimastix frontalis.*

Xylanases can also hydrolyse higher xylo-oligosaccharides, the affinity for such substrates increasing with increasing degree of polymerization (DP). By contrast with the β-xylosidases, they do not cleave xylobiose and most such enzymes investigated, including representatives from *Aspergillus niger, Aspergillus ochraeus, Bacillus subtilis, Humicola grisea* var *thermoidea* and *Streptomyces lividans,* do not hydrolyse aryl β-D-xylosides. Again, however, some exceptions have been noted. Among these are Xyn A from *Caldocellum saccharolyticum*, a xylanase from *Cryptococcus albidus* and one from *Thermoascus aurantiacus,* whereas Xyl 1 from *Streptomyces lividans* can hydrolyse *p*-nitrophenyl (PNP β-D-glucoside) but has no action against PNP β-D-glucoside. In the case of the *C. albidus* enzyme, it has been established that conversion of PNP β-D-glucoside occurs, not by direct hydrolysis, but by a complex transfer reaction sequence (see review by Tuohy *et al* 1993a).

Because of their potential applications, the enzyme systems that degrade xylans have been in the subject of much study (see Wong *et al.,* 1988; Puls & Poutanen. 1989). The complete enzyme systems or the side-chain cleaving activities, as appropriate, may be used in the hydrolysis of xylans to their monomeric constituents for subsequent fermentation to ethanol or xylitol, in the bleaching of pulp, in the preparation of clean cellulose, in silage making and in the preparation of modified hemicelluloses (see for example, Biely, 1985; Wong *et al.,* 1988).

Xylanase enxymes have been combined with other protease enzymes and added to animal feeds to enhance the digestion of nutrients (see WO 96/05739). Thermostable xylanases (from, for example, *Microtetraspora flesuosa* and *Thermomonospora fusca*) have also been added to animal feeds to enable animals digest the feeds more efficiently (see WO 95/29997). However, the addition of these xylanase enzymes has not been consistenly effective. It is thought that naturally occuring xylanase inhibitors from cereals, such as wheat, can pose problems especially when xylanase enzymes are added

to chicken feed with a high content of ground wheat. By way of example, it has been suggested that some inhibitors from wheat may be resistant to temperatures of from about 80°C to 95°C. In this regard, variations in temperature during the pelleting process may mean that the naturally occurring xylanase inhibitors are not completely denatured. This may have an adverse effect on the efficacy of added xylanase enzymes.

If such temperature mediated denaturing of the naturally occuring xylanase inhibitors does not occur during animal feed processing, it is of course extremely disadvantageous as then the xylanase enzyme, even if it is an thermostable xylanase enzyme, will not give rise to the effect for which it was added, or will only give rise to such an effect to a limited extent. One possible way of overcoming this problem would be to include significantly greater relative amounts of the thermostable xylanase enzyme to the food and/or feed in order to compensate for the deactivation of a certain proportion of the xylanase enzyme. However, adding such additional amounts of enzyme is disadvantageous from an economic viewpoint.

The present invention thus seeks to overcome the problems associated with the preparation of food and/or feed supplements comprising naturally occuring xylanase inhibitors from cereals such as wheat.

SUMMARY ASPECTS OF THE INVENTION

According to the present invention, there is provided a thermostable xylanase enzyme. The activity of this xylanase enzyme is substantially independent of any level of a wheat xylanase inhibitor. This thermostable xylanase enzyme can be used in a food and/or feed supplement to modifying a xylan polymer present in that supplement.

The thermostable xylanase enzyme of the present invention has relatively low specific endo-xylanase activity. Consquently, this xylanase was not an obvious choice for a new food and/or feed xylanase as this enzyme is very different from the xylanase activity of traditional food and/or feed xylanase enzymes which have high specific endo-xylanase activity.

Unexpectedly and advantageously, the novel thermostable xylanase enzyme of the present invention allows the preparation of food and/or feed supplement at high temperatures without affecting the performance of the xylanase enzyme as the activity of this enzyme is substantially independent of any wheat xylanase inhibitors which may be present in a cereal based food and/or feed supplement.

This endo-xylanase activity of the enzyme of the present invention is also advantageous as the thermostable xylanase enzyme is capable of delivering low molecular weight arabinoxylan fragments *in vivo* which may have a profound positive influence on intestinal microflora. In contrast, most of the other known endo-xylanase enzymes produce high molecular weight fragments.

DETAILED ASPECTS OF THE INVENTION

In one embodiment, the present invention provides a thermostable xylanase enzyme capable of modifying a xylan polymer in a food and/or feed supplement wherein the activity of the xylanase enxyme is substantially independent of any level of a wheat xylanase inhibitor that may be present in the food and/or feed supplement.

Other aspects of the present invention are presented in the accompanying claims and in the following description and discussion. These aspects are presented under separate section headings. However, it is to be understood that the teachings under each section heading are not necessarily limited to that particular section heading.

XYLANASE

As used herein, the term "xylanase" refers to a hemicellulase enzyme that cuts through the β-1,4 bonds within the xylosidic chain of a xylan polymer. Xylanase enzymes can be classified as endo-xylanases or exo-xylanases depending on whether they cleave an internal β-1,4 linkage or a β-1,4 linkage at the non-reducing end of the xylan polymer.

The xylanase enzyme of the present invention is a thermostable xylanase enzyme which is herein after referred to as a themostable TX-1 xylanase enzyme.

THERMOSTABLE XYLANASE

Preferably the thermostable xylanase enzyme of the present invention is derivable from a fungus.

Preferably the thermostable TX-1 xylanase enzyme of the present invention is derivable from *Talaromyces emersonii.*

By way of background, *Talaromyces emersonii* produces a complete xylan-degrading enzyme system when grown on the appropriate inducing substrates (Filho *et al.,* 1989: Tuohy & Coughlan, 1992). Hydrolysis experiments with crude preparations provide evidence for the existence of endoxylanase, β-xylosidase, α-arabinofuranosidase, α-glucuronidase, acetylferuloyl-and coumaroyl-esterases. The participation of these enzyms in xylan degradation has been confirmed by independent assays. Preliminary work has indicated the existence of multiple forms of xylan-degrading enzymes (Tuohy & Coughlan, 1992). Subsequent studies have shown that the system includes at least 13 endo-β-1,4-xylanases (EC 3.2.1.8), one β-xylosidase (EC 3.2.1.37), one α-L-arabinofuranosidase (EC 3.2.1.55), three acetylxylan esterases, and have confirmed the existence of a α-glucuronidase, ferulic and p-coumaric acid esterase activies. Most of these enzymes have been purified to apparent homogeneity and charaterized as individual components or combinations thereof. The physiochemical characteristics of these xylan-degrading enzyme purified thus far include M_r values ranging from 30,000-131 000 and pIvalues from pH 3.8-5.3. According to the literature, most of the fungal and bacterial xylanases have M_r values in the range 8500-85 000, with pI values ranging from 4.0 to 10.3. Two endoxylanases (one high M_r and one low M_r) purified from A. *niger* filtrates are good examples of this theory (Coughlan *et al.,* 1993). However, exceptions to this rule have been found, for example in *Trichoderma pseudokoningii* (Baker *et al.,* 1997). Endoxylanases are almost exclusively single subunit proteins. However, Xyl II from *T. emersonii* differs in being a dimeric protein. This is not the

only unusual feature this particular enzyme possesses. Generally, multiple isoenzymic forms of xylanases have been found in the culture filtrates of fungi. *Talaromyces emersonii* has been shown to be no exception. Dekker and Richards (1976) proposed that the complexity of xylans required the action of multiple xylanases with overlapping yet different specificities to effect extensive hydrolysis. Some of this multiplicity has been demonstrated to be genetically determined. However, post-translational modification, such as differential glycosylation, proteolysis, or aggregation with other polysaccharides may also account for some of this multiplicity. Other fungi known to produce comparative numbers of xylanase enzymes to those found in *T, emersonii* filtrates, are *Aspergillus niger* (15 endoxylanases) Biely *et al.*, 1985) and *Trichoderma viride* (13 endoxylanases)(Biely *et al.*, 1985). The endoxlanases from *T. emersonii* are glycoproteins and have been shown to have high temperature optima (67-80°C) and high thermal stabilities. All the isoenzymes have acidic pH optima (pH 2.5-4.7 (Tuohy *et al.*, 1993 *a.b*). Thermostable enzymes offer particular advantages in many industrial applications. All but two of the endoxylanases, namely Xyl II and Xyl III, are active against a variety of xylans. Examination of the products of hydrolysis of a number of different xylans indicated that each enzyme has different specificities (Tuohy *et al.*,1993b). Transferase activity was shown to occur with a number of these enzymes, exceptions were XynII, Xyn IV and Xyn IX. K_m values, with the soluble fraction of at spelts xylan as substrate, range from 0.4-13.3 mg/ml and k_{cat} *values from 216-9213/s (Tuohy et al.*, 1993b. Hydrolysis of unsubstituted xylooligomers demonstrated that affinity increased with increasing degree of polymerisation (DP). Only Xyl II and Xyl III displayed activity against artificial xylosides. These two enzymes were found to be very specific and unusual xylanases. Both enzymes catalyzed the hydrolysis of artificial xylosides, xylooligosaccharides and unsubstituted xylans. Both displayed no activity against a variety of substituted xylans. Furthermore, Xyl II seemed to behave like a xylobiosidase (analogous to cellobiosidase) liberating X_2 exclusively from $pNPX_2$. The action of this enzyme on Lenzing xylan (DP30) resulted in rapid and complete degradation of the polymeric material with 3 h to X_2 and X_3. Prolonged hydrolysis yielded X_2 as the major end-product. Preincubation of wheat straw arabinoxylan with arabinofuranosidase to remove side-chain arabinose groups, facilitated access of these enzymes to the polymer backbone with concomitant rapid and extensive hydrolysis (see

Fig. 1) (Tuohy *et al.*, 1993a). Xylotriose (X_3) was the major end-product produced by XylI (see Table 5 of Tuohy *et al* 1993a Biochem J 290; 515-23). In contrast, Table 2 shows that Xylobiose (X_2) is the major end product of the thermostable TX-1 xylanase of the present invention. A comparison of the physical properties of different xylanases derivable from *Talaromyces emerzonii* is outlined in Table 1 below.

Table 1

	Xyl I	Xyl II	Xyl III	Xylanase**	TX-1
Mr(SDS)	97,500	74,850	54,200	nd	38,500
pI	8.9	5.3	4.2	nd	4.5*
optimum pH	2.5	4.2	3.5	5.0	3.0
optimum temperature	60°C	78°C	67°C	87°C	80°C

Xyl I, Xyl II and Xyl III: in Tuohy M G *et al.* (1993a). The xylan-degrading enzyme system of *Talaromyces emerzonii*; novel enzymes with activity against aryl-β-D-xylosides and unsubstituted xylans, *Biochem. J.*290, 515-23.
**Xylanase: in GB 2150933
* theoretical pI determination

TX-1 XYLANASE CHARACTERISATION

The novel thermostable xylanase enzyme (TX-1) of the present invention is characterised by a molecular weight of 38, 500 Daltons; a pH optimum of 3.0; thermostability at temperatures from about 70°C to about 95°C; a temperature optimum of 80°C; a thoeretical pI of about 4.5; a xylobiose (X_2) end product; and a retention of capability to modify a xylan polymer even when naturally occuring wheat xylanase inhibitors are present in the food and/or feed supplement.

XYLANASE INHIBITORS

As used herein, the term "xylanase inhibitor" refers to a protein whose role is to control the depolymerisation of complex carbohydrates, such as arabinoxylan, found in plant cell walls. These xylanase inhibitors are capable of reducing the activity of naturally occuring xylanase enzymes as well as those of fungal or bacterial origin. Although the presence of xylanase inhibitors have been reported in cereal seeds (see for example McLauchlan *et al* 1999; Rouau and Suget 1998) their impact on the efficacy of xylanase

enzymes and in particular thermostable xylanase enzyme which have been added to food and/or feed supplements, has not been extensively examined.

By way of example, the xylanases (and specifically, the endo-β-1,4-xylanases) which are produced by certain bacteria, fungi and plants and which hydrolyse the β-1, 4-xylan linkages in the xylan component of plant cell walls have been grouped into two classes, family 10 (also called F) and family 11 (also called G). In this regard, the TX-1 thermostable xylanase enzyme of the present invention is regarded as a member of the family 10 xylanases and is not regarded as a member of the family 11 xylanases.

In relation to studies on inhibition of xylanases enzymes from these two families, McLauchlan *et al* (1999a) disclose the isolation and characterisation of a protein from wheat that binds to and inhibits two family-11 xylanases. Likewise, WO 98/49278 demonstrates the effect of a wheat flour extract on the activity of a group of microbial xylanases all of which are classified as family 11 xylanases. Debyser *et al* (1999) also disclose that endoxylanases from *Aspergillus niger* and *Bacillus subtilis*, which are both members of the family 11 xylanases were inhibited by a wheat xylanase inhibitor called TAXI. Although the endoxylanases from *Aspergillus aculeatus* and that from a rumen micro-organism, which are members of the family 10 xylanases were not inhibited in the assay method of Debyser et al (1999), McLauchlan *et al* (1999b) teaches that extracts from commercial flours such as wheat, barley, rye and maize are capable of inhibiting both family 10 and 11 xylanases. Thus, there are conflicting reports on xylanase inhibitors and their effects on family 10 and family 11 xylanases.

Consequently, the present invention demonstrates the highly surprising finding that the thermostable TX-1 xylanase enzyme of the present invention is effective in modifying a xylan present in a food and/or feed supplement even when naturally occuring xylanase inhibitors are present in a food and/or feed supplement. In other words, the present invention demonstrates that the activity of the TX-1 xylanase enzyme is substantially independent of naturally occuring xylanase inhibitors derivable from cereal seeds, particularly naturally occuring xylanase inhibitors derivable from wheat based cereal seeds.

In another embodiment, the present invention provides a thermostable TX-1 xylanase enzyme capable of modifying a xylan polymer in a food and/or feed supplement.

MODIFYING

As used herein, the term "modifying" or "modified" refers to a xylan polymer, such as arabinoxylan, which is degraded by a xylanase enzyme to produce low molecular weight (mostly xylobiose, X_2) fragments.

XYLAN

As used herein, the term "xylan" refers to a polymer of D-xylose residues that are joined through β-1,4 linkages and which have various substituent groups. These xylans can include but are not limited to xylans from cereals and grasses such as arabinoxylan and glucuronoxylan. The term "xylan" is often used interchangeably with the term "xylan polymer".

Preferably the xylan is a cereal or derivable from a cereal.

CEREAL

As used herein, the term "cereal" means any kind of grain used for food and/or any grass producing this grain such as but not limited to any one of wheat, milled wheat, barley, maize, sorghum, rye, oats, triticale and rice or combinations thereof.

In one preferred embodiment, the cereal is a wheat cereal or a legume (such as for example pea or soy legumes).

The xylan in the food and/or feed supplement of the present invention is modified by contacting the xylan with the novel thermostable xylanase enzyme (TX-1) of the present invention.

CONTACTING

As used herein, the term "contacting" includes but is not limited to spraying, coating, impregnating or layering the food and/or feed supplement with the TX-1 enzyme of the present invention.

In one embodiment, the food and/or feed supplement of the present invention may be prepared by mixing the TX-1 xylanase enzyme directly with a food and/or feed supplement. By way of example, the TX-1 xylanase enzyme may be contacted (for example, by spraying) onto a cereal-based food and/or feed supplement such as milled wheat, maize or soya flour.

It is also possible to incorporating the TX-1 xylanase enzyme it into a second (and different) food and/or feed or drinking water which is then added to the food and/or feed supplement of the present invention. Accordingly, it is not essential that the TX-1 xylanase enzyme provided by the present invention is incorporated into the cereal-based food and/or feed supplement itself, although such incorporation forms a particularly preferred aspect of the present invention.

FOOD AND/OR FEED SUPPLEMENT COMBINATIONS

In one embodiment of the present invention, the food and/or feed supplement may be combined with other food and/or feed components to produce a cereal-based food and/or feed. Such other food and/or feed components may include one or more other (preferably thermostable) enzyme supplements, vitamin food and/or feed supplements, mineral food and/or feed supplements and amino acid food and/or feed supplements. The resulting (combined) food and/or feed supplement comprising possibly several different types of compounds can then be mixed in an appropriate amount with the other food and/or feed components such as cereal and protein supplements to form a human food and/or an animal feed.

ENZYME COMBINATION

In one preferred embodiment, the food and/or feed supplement of the present invention can be prepared by mixing different enzymes having the appropriate activities to produce an enzyme mix. By way of example, a cereal-based food and/or feed supplement formed from e.g. milled wheat or maize may be contacted (eg by spraying) either simultaneously or sequentially with the xylanase enzyme and other enzymes having appropriate activities. These enzymes may include but are not limited to any one or more of an amylase, a glucoamylase, a pectinase, a mannanase, an a galactosidase, a phytase, a lipase, a P-glucanase, an-arabinofuranosidase, an amylase, a pectinase and a xylanase. Enzymes having the desired activities may for instance be mixed with the xylanase of the present invention either before contacting these enzymes with a cereal-based food and/or feed supplement or alternatively such enzymes may be contacted simultaneously or sequentially on such a cereal based supplement. The food and/or feed supplement is then in turn mixed with a cereal-based food and/or feed to prepare the final food and/or feed. It is also possible to formulate the food and/or feed supplement as a solution of the individual enzyme activities and then mix this solution with a food and/or feed material prior to processing the food and/or feed supplement into pellets or as a mash.

XYLANASE PRODUCTION

The thermostable TX-1 xylanase enzyme can be obtainable from or produced by any suitable source, whether natural or not, or it may be a synthetic thermostable TX-1 xylanase enzyme, a semi-synthetic thermostable TX-1 xylanase enzyme, a mimetic, a derivatised thermostable TX-1 xylanase enzyme, a recombinant thermostable TX-1 xylanase enzyme, a fermentation optimised enzyme, a fusion protein or equivalents, mutants and derivatives thereof as long as it retains the required activity of the thermostable TX-1 xylanase enzyme of the present invention.

The term "mimetic" relates to any chemical which may be a peptide, polypeptide, antibody or other organic chemical which has the same activity as the thermostable TX-1 xylanase enzyme of the present invention.

The term "derivative" or "derivatised" as used herein includes chemical modification of an thermostable TX-1 xylanase enzyme. Illustrative of such modifications would be replacement of hydrogen by an alkyl, acyl, or amino group. Preferably, the non-native thermostable TX-1 xylanase enzyme includes at least a portion of which has been prepared by recombinant DNA techniques or produced by chemical synthesis techniques or combinations thereof.

Preferably, the non-native thermostable TX-1 xylanase enzyme is prepared by the use of chemical synthesis techniques.

The thermostable TX-1 xylanase enzyme of the present invention or variants, homologues, derivatives, fragments or mimetics thereof may be produced using chemical methods to synthesize the thermostable TX-1 xylanase enzyme amino acid sequence, in whole or in part. For example, peptides can be synthesized by solid phase techniques, cleaved from the resin, and purified by preparative high performance liquid chromatography (e.g., Creighton (1983) Proteins Structures And Molecular Principles, WH Freeman and Co, New York NY). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; Creighton, *supra*).

Direct synthesis of the thermostable TX-1 xylanase enzyme or variants, homologues, derivatives, fragments or mimetics thereof can be performed using various solid-phase techniques (Roberge JY *et al* (1995) Science 269: 202-204) and automated synthesis may be achieved, for example, using the ABI 43 1 A Peptide Synthesizer (Perkin Elmer) in accordance with the instructions provided by the manufacturer. Additionally, the amino acid sequences obtainable from the thermostable TX-1 xylanase enzyme, or any part thereof, may be altered during direct synthesis and/or combined using chemical

methods with a sequence from other subunits, or any part thereof, to produce a variant thermostable TX-1 xylanase enzyme.

Preferably the thermostable TX-1 xylanase enzyme of the present invention comprises the amino acid sequence set out in SEQ ID No 1 (see Figure 10) which is derivable from *T. emersonii.*

AMINO ACID SEQUENCE

As used herein, the term "amino acid sequence" refers to peptide, polypeptide sequences, protein sequences or portions thereof.

The thermostable TX-1 xylanase enzyme of the present invention may be in a substantially isolated form. It will be understood that the protein may be mixed with carriers or diluents which will not interfere with the intended purpose of the thermostable TX-1 xylanase enzyme and still be regarded as substantially isolated. The thermostable TX-1 xylanase enzyme of the present invention may also be in a substantially purified form, in which case it will generally comprise the thermostable TX-1 xylanase enzyme in a preparation in which more than 90%, e.g. 95%, 98% or 99% of the thermostable TX-1 xylanase enzyme in the preparation is a peptide comprising SEQ ID No 1 or variants, homologues, derivatives or fragments thereof.

VARIANTS /HOMOLOGUES/DERIVATIVES OF AMINO ACID SEQUENCES

Preferred amino acid sequences of the present invention are set out in SEQ ID No 1 or are sequences obtainable from the thermostable TX-1 xylanase enzyme of the present invention but also include homologous sequences obtained from any source, for example related viral/bacterial proteins, cellular homologues and synthetic peptides, as well as variants or derivatives thereof.

Thus, the present invention covers variants, homologues or derivatives of the amino acid sequences presented herein, as well as variants, homologues or derivatives of the nucleotide sequence coding for those amino acid sequences.

In the context of the present invention, a homologous sequence is taken to include an amino acid sequence which is at least 75, 85 or 90% identical, preferably at least 95 or 98% identical at the amino acid level over at least, for example, the amino acid sequence as set out in SEQ ID No 1 of the sequence listing herein. In particular, homology should typically be considered with respect to those regions of the sequence known to be essential for enzyme activity (such as amino acids at positions) rather than non-essential neighbouring sequences. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

% homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising

unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package (see below) the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A.; Devereux *et al.*, 1984, Nucleic Acids Research 12:387). Examples of other software than can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel *et al.*, 1999 *ibid* – Chapter 18), FASTA (Atschul *et al.*, 1990, J. Mol. Biol., 403-410) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel *et al.*, 1999 *ibid*, pages 7-58 to 7-60). However it is preferred to use the GCG Bestfit program.

Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default

values or a custom symbol comparison table if supplied (see user manual for further details). It is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

The terms "variant" or "derivative" in relation to the amino acid sequences of the present invention includes any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acids from or to the sequence providing the resultant amino acid sequence has an enzyme activity, preferably having at least the same enzyme activity as the amino acid sequence set out in SEQ ID No 1.

SEQ ID No 1 may be modified for use in the present invention. Typically, modifications are made that maintain the enzyme activity of the sequence. Amino acid substitutions may be made, for example from 1, 2 or 3 to 10 or 20 substitutions provided that the modified sequence retains the required enzyme activity. Amino acid substitutions may include the use of non-naturally occurring analogues.

The thermostable TX-1 xylanase enzyme of the present invention may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent enzyme. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the enzyme activity of the thermostable TX-1 xylanase enzyme is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.

Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R
AROMATIC		H F W Y

Preferably, the non-native thermostable TX-1 xylanase enzyme has been prepared by use of recombinant techniques.

VARIANTS/HOMOLOGUES/DERIVATIVES OF NUCLEOTIDE SEQUENCES

It will be understood by a skilled person that numerous different nucleotide sequences can encode the same thermostable TX-1 xylanase enzyme of the present invention as a result of the degeneracy of the genetic code. In addition, it is to be understood that skilled persons may, using routine techniques, make nucleotide substitutions that do not affect the thermostable TX-1 xylanase enzyme encoded by the nucleotide sequence of the present invention to reflect the codon usage of any particular host organism in which the thermostable TX-1 xylanase enzyme of the present invention is to be expressed.

The terms "variant", "homologue" or "derivative" in relation to the nucleotide sequence set out in SEQ ID No 2 (see Figure 11) or SEQ ID No 3 (see Figure 12) of the present invention includes any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid from or to the sequence providing the

resultant nucleotide sequence codes for a thermostable TX-1 xylanase enzyme having an enzyme activity, preferably having at least the same activity as the nucleotide sequence set out in SEQ ID No 2 or SEQ ID No 3 of the sequence listings.

As indicated above, with respect to sequence homology, preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to the sequences shown in the sequence listing herein. More preferably there is at least 95%, more preferably at least 98%, homology. Nucleotide homology comparisons may be conducted as described above. A preferred sequence comparison program is the GCG Wisconsin Bestfit program described above. The default scoring matrix has a match value of 10 for each identical nucleotide and -9 for each mismatch. The default gap creation penalty is -50 and the default gap extension penalty is -3 for each nucleotide.

The present invention also encompasses nucleotide sequences that are capable of hybridising selectively to the sequences presented herein, or any variant, fragment or derivative thereof, or to the complement of any of the above. Nucleotide sequences are preferably at least 15 nucleotides in length, more preferably at least 20, 30, 40 or 50 nucleotides in length.

HYBRIDISATION

The term "hybridization" as used herein shall include "the process by which a strand of nucleic acid joins with a complementary strand through base pairing" as well as the process of amplification as carried out in polymerase chain reaction (PCR) technologies.

Nucleotide sequences of the invention capable of selectively hybridising to the nucleotide sequences presented herein, or to their complement, will be generally at least 75%, preferably at least 85 or 90% and more preferably at least 95% or 98% homologous to the corresponding nucleotide sequences presented herein over a region of at least 20, preferably at least 25 or 30, for instance at least 40, 60 or 100 or more contiguous nucleotides. Preferred nucleotide sequences of the invention will comprise regions homologous to the nucleotide sequence set out in SEQ ID No 2 or SEQ ID No 3 preferably

at least 80 or 90% and more preferably at least 95% homologous to the nucleotide sequence set out in SEQ ID No 2 or SEQ ID No 3.

The term "selectively hybridizable" means that the nucleotide sequence used as a probe is used under conditions where a target nucleotide sequence of the invention is found to hybridize to the probe at a level significantly above background. The background hybridization may occur because of other nucleotide sequences present, for example, in the cDNA or genomic DNA library being screened. In this event, background implies a level of signal generated by interaction between the probe and a non-specific DNA member of the library which is less than 10 fold, preferably less than 100 fold as intense as the specific interaction observed with the target DNA. The intensity of interaction may be measured, for example, by radiolabelling the probe, e.g. with ^{32}P.

Hybridization conditions are based on the melting temperature (Tm) of the nucleic acid binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego CA), and confer a defined "stringency" as explained below.

Maximum stringency typically occurs at about Tm-5°C (5°C below the Tm of the probe); high stringency at about 5°C to 10°C below Tm; intermediate stringency at about 10°C to 20°C below Tm; and low stringency at about 20°C to 25°C below Tm. As will be understood by those of skill in the art, a maximum stringency hybridization can be used to identify or detect identical nucleotide sequences while an intermediate (or low) stringency hybridization can be used to identify or detect similar or related polynucleotide sequences.

In a preferred aspect, the present invention covers nucleotide sequences that can hybridise to the nucleotide sequence of the present invention under stringent conditions (e.g. 65°C and 0.1xSSC {1xSSC = 0.15 M NaCl, 0.015 M Na_3 Citrate pH 7.0). Where the nucleotide sequence of the invention is double-stranded, both strands of the duplex, either individually or in combination, are encompassed by the present invention. Where the nucleotide

sequence is single-stranded, it is to be understood that the complementary sequence of that nucleotide sequence is also included within the scope of the present invention.

Nucleotide sequences which are not 100% homologous to the sequences of the present invention but fall within the scope of the invention can be obtained in a number of ways. Other variants of the sequences described herein may be obtained for example by probing DNA libraries made from a range of sources. In addition, other viral/bacterial, or cellular homologues particularly cellular homologues found in mammalian cells (e.g. rat, mouse, bovine and primate cells), may be obtained and such homologues and fragments thereof in general will be capable of selectively hybridising to the sequences shown in the sequence listing herein. Such sequences may be obtained by probing cDNA libraries made from or genomic DNA libraries from other animal species, and probing such libraries with probes comprising all or part of the nucleotide sequence set out in SEQ I.D. No 2 or SEQ ID No 3 under conditions of medium to high stringency. Similar considerations apply to obtaining species homologues and allelic variants of the amino acid and/or nucleotide sequences of the present invention.

Variants and strain/species homologues may also be obtained using degenerate PCR which will use primers designed to target sequences within the variants and homologues encoding conserved amino acid sequences within the sequences of the present invention. Conserved sequences can be predicted, for example, by aligning the amino acid sequences from several variants/homologues. Sequence alignments can be performed using computer software known in the art. For example the GCG Wisconsin PileUp program is widely used. The primers used in degenerate PCR will contain one or more degenerate positions and will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences.

Alternatively, such nucleotide sequences may be obtained by site directed mutagenesis of characterised sequences, such as the nucleotide sequence set out in SEQ ID. No 2 or SEQ ID No 3. This may be useful where for example silent codon changes are required to sequences to optimise codon preferences for a particular host cell in which the nucleotide sequences are being expressed. Other sequence changes may be desired in order to

introduce restriction enzyme recognition sites, or to alter the enzyme activity of the thermostable TX-1 xylanase enzyme encoded by the nucleotide sequences.

The nucleotide sequences of the present invention may be used to produce a primer, e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels, or the nucleotide sequences may be cloned into vectors. Such primers, probes and other fragments will be at least 15, preferably at least 20, for example at least 25, 30 or 40 nucleotides in length, and are also encompassed by the term nucleotide sequence of the invention as used herein.

The nucleotide sequences such as a DNA polynucleotides and probes according to the invention may be produced recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by standard techniques.

In general, primers will be produced by synthetic means, involving a step wise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

Longer nucleotide sequences will generally be produced using recombinant means, for example using a PCR (polymerase chain reaction) cloning techniques. This will involve making a pair of primers (e.g. of about 15 to 30 nucleotides) flanking a region of the targeting sequence which it is desired to clone, bringing the primers into contact with mRNA or cDNA obtained from an animal or human cell, performing a polymerase chain reaction (PCR) under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector

Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence, may be used to

clone and express the thermostable TX-1 xylanase enzyme. As will be understood by those of skill in the art, it may be advantageous to produce the thermostable TX-1 xylanase enzyme - encoding nucleotide sequences possessing non-naturally occurring codons. Codons preferred by a particular prokaryotic or eukaryotic host (Murray E *et al* (1989) Nuc Acids Res 17:477-508) can be selected, for example, to increase the rate of the thermostable TX-1 xylanase enzyme expression or to produce recombinant RNA transcripts having desirable properties, such as a longer half-life, than transcripts produced from naturally occurring sequence.

In an alternative embodiment of the invention, the coding sequence of the thermostable TX-1 xylanase enzyme or variants, homologues, derivatives, fragments or mimetics thereof may be synthesized, in whole or in part, using chemical methods well known in the art (see Caruthers MH *et al* (1980) Nuc Acids Res Symp Ser 215-23, Horn T *et al* (1980) Nuc Acids Res Symp Ser 225-232).

VECTORS

The nucleotide sequences of the present invention can be incorporated into a recombinant replicable vector. The vector may be used to replicate the nucleotide sequence in a compatible host cell. Thus in a further embodiment, the invention provides a method of making the thermostable TX-1 xylanase enzyme of the present invention by introducing a nucleotide sequence of the present invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell.

Preferably, a nucleotide sequence of present invention which is inserted into a vector is operably linked to a control sequence that is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector.

Control sequences operably linked to sequences encoding the protein of the invention include promoters/enhancers and other expression regulation signals. These control

sequences may be selected to be compatible with the host cell in which the expression vector is designed to be used. The control sequences may be modified, for example by the addition of further transcriptional regulatory elements to make the level of transcription directed by the control sequences more responsive to transcriptional modulators.

The term "operably linked" means that the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences.

The vectors of the present invention may be transformed or transfected into a suitable host cell as described below to provide for expression of an thermostable TX-1 xylanase enzyme of the present invention. This process may comprise culturing a host cell transformed with an expression vector as described above under conditions to provide for expression by the vector of a coding sequence encoding the thermostable TX-1 xylanase enzyme, and optionally recovering the expressed protein.

The vectors may be for example, plasmid or virus vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin resistance gene for a mammalian vector.

PROMOTERS

The term promoter is well-known in the art and encompasses nucleic acid regions ranging in size and complexity from minimal promoters to promoters including upstream elements and enhancers.

The promoter is typically selected from promoters which are functional in mammalian, cells, although prokaryotic promoters and promoters functional in other eukaryotic cells

may be used. The promoter is typically derived from promoter sequences of viral or eukaryotic genes. For example, it may be a promoter derived from the genome of a cell in which expression is to occur. With respect to eukaryotic promoters, they may be promoters that function in a ubiquitous manner (such as promoters of α-actin, β-actin, tubulin) or, alternatively, a tissue-specific manner (such as promoters of the genes for pyruvate kinase). Tissue-specific promoters specific for may be particularly preferred. They may also be promoters that respond to specific stimuli, for example promoters that bind steroid hormone receptors. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR) promoter, the rous sarcoma virus (RSV) LTR promoter or the human cytomegalovirus (CMV) IE promoter.

It may also be advantageous for the promoters to be inducible so that the levels of expression of the heterologous gene can be regulated during the life-time of the cell. Inducible means that the levels of expression obtained using the promoter can be regulated.

In addition, any of these promoters may be modified by the addition of further regulatory sequences, for example enhancer sequences. Chimeric promoters may also be used comprising sequence elements from two or more different promoters described above.

TX-1 CONSTRUCTS

The thermostable TX-1 xylanase enzyme produced by a host recombinant cell may be secreted or may be contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing the thermostable TX-1 xylanase enzyme coding sequences can be designed with signal sequences which direct secretion of the thermostable TX-1 xylanase enzyme coding sequences through a particular prokaryotic or eukaryotic cell membrane. Other recombinant constructions may join the thermostable TX-1 xylanase enzyme coding sequence to nucleotide sequence encoding a polypeptide domain which will facilitate

purification of soluble proteins (Kroll *DJ et al* (1993) DNA Cell Biol 12:441-53). Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals (Porath J (1992) Protein Expr Purif 3 -.26328 1), protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp, Seattle, WA). The inclusion of a cleavable linker sequence such as Factor XA or enterokinase (Invitrogen, San Diego, CA) between the purification domain and the thermostable TX-1 xylanase enzyme is useful to facilitate purification.

The thermostable TX-1 xylanase enzyme of the invention may also be produced as fusion proteins, for example to aid in extraction and purification. Examples of fusion protein partners include glutathione-S-transferase (GST), 6xHis, GAL4 (DNA binding and/or transcriptional activation domains) and β-galactosidase. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences. Preferably the fusion protein will not hinder the secondary binding activity of the thermostable TX-1 xylanase enzyme comprising the amino acid sequence of the present invention.

The present invention thus provides for fusion proteins comprising the respected target or an enzymatically active fragment or derivative thereof linked to an affinity tag such as glutathione-S-transferase (GST), biotin, His6, *myc* and hemagglutinin (HA) (as described in Wilson *et al* (1984 Cell 37 767). Preferably the fused recombinant protein comprises an antigenic co-protein such as GST, β-galactosidase or the lipoprotein D from *Haemophilus influenzae* which are relatively large co-proteins, which solubilise and facilitate production and purification thereof. Alternatively, the fused protein may comprise a carrier protein such as bovine serum albumin (BSA) or keyhole limpet haemocyanin (KLH). In certain embodiments of the present invention, the marker sequence is a hexa-histidine peptide, as provided in the pQE vector (Qiagen Inc) and described in Gentz *et al* (1989 PNAS 86: 821-824). Such fusion proteins are readily expressable in yeast culture (as described in Mitchell *et al* 1993 Yeast 5: 715-723) and

are easily purified by affinity chromatography. A fusion protein may also be engineered to contain a cleavage site located between the nucleotide sequence encoding the thermostable TX-1 xylanase enzyme and the heterologous protein sequence, so that the thermostable TX-1 xylanase enzyme may be cleaved and purified away from the heterologous moiety. In another embodiment, an assay for the target protein may be conducted using the entire, bound fusion protein. Alternatively, the co-protein may act as an adjuvant in the sense of providing a generalised stimulation of the immune system. The co-protein may be attached to either the amino or carboxy terminus of the first protein.

In another embodiment of the invention, an thermostable TX-1 xylanase enzyme natural, modified or recombinant sequence may be ligated to a heterologous sequence to encode a fusion protein. For example, for screening of peptide libraries for inhibitors of the thermostable TX-1 xylanase enzyme activity, it may be useful to encode a chimeric thermostable TX-1 xylanase enzyme expressing a heterologous epitope that is recognized by a commercially available antibody.

Although the presence/absence of marker gene expression suggests that the nucleotide sequence and/or its thermostable TX-1 xylanase enzyme is also present, its presence and expression should be confirmed. For example, if the thermostable TX-1 xylanase enzyme coding sequence is inserted within a marker gene sequence, recombinant cells containing the thermostable TX-1 xylanase enzyme coding regions may be identified by the absence of marker gene function. Alternatively, a marker gene may be placed in tandem with a the thermostable TX-1 xylanase enzyme coding sequence under the control of a single promoter. Expression of the marker gene in response to induction or selection usually indicates expression of the thermostable TX-1 xylanase enzyme as well.

Additional methods to quantitate the expression of a particular molecule include radiolabeling (Melby PC *et al* 1993 J Immunol Methods 159:235-44) or biotinylating (Duplaa C *et al* 1993 Anal Biochem 229-36) nucleotides, coamplification of a control nucleic acid, and standard curves onto which the experimental results are interpolated.

Quantitation of multiple samples may be speeded up by running the assay in an ELISA format where the oligomer of interest is presented in various dilutions and a spectrophotometric or calorimetric response gives rapid quantitation.

VECTOR TRANSFER

Vectors comprising the nucleotide sequences of the present invention may be introduced into host cells for the purpose of replicating the vectors comprising the nucleotide sequences of the present invention and/or expressing the proteins of the invention encoded by the nucleotide sequences of the present invention.

Vectors comprising nucleotide sequences of the present invention may introduced into suitable host cells using a variety of techniques known in the art, such as transfection, transformation and electroporation. Where the vectors comprising the nucleotide sequences of the present invention are to be administered to animals, several techniques are known in the art, for example infection with recombinant viral vectors such as retroviruses, herpes simplex viruses and adenoviruses, direct injection of nucleic acids and biolistic transformation.

Uptake of naked nucleic acid constructs by mammalian cells is enhanced by several known transfection techniques for example those including the use of transfection agents. Example of these agents include cationic agents (for example calcium phosphate and DEAE-dextran) and lipofectants (for example lipofectam™ and transfectam™). Typically, nucleic acid constructs are mixed with the transfection agent to produce a composition.

The vectors comprising nucleotide sequences encoding thermostable TX-1 xylanase enzymes of the present invention for use in affecting viral infections may be administered directly as a naked nucleic acid construct, preferably further comprising flanking sequences homologous to the host cell genome. When the vectors comprising the nucleotide sequences of the present invention are administered as a naked nucleic

acid, the amount of nucleic acid administered may typically be in the range of from 1 μg to 10 mg, preferably from 100 μg to 1 mg.

In one embodiment of the present invention, the nucleotide sequence of the present invention may be inserted into, for example, plasmid pMM68, which is then used to transfect host cells, such as *Vibrio sp.60.*

HOST CELLS

Host cells comprising nucleotide sequences of the present invention may be used to express the thermostable TX-1 xylanase enzymes of the present invention. Although the proteins of the invention may be produced using prokaryotic cells as host cells, it is preferred to use eukaryotic cells, for example yeast, insect or mammalian cells, in particular mammalian cells. Suitable host cells include bacteria such as *E. coli*, yeast, mammalian cell lines and other eukaryotic cell lines, for example insect Sf9 cells.

Host cells may be cultured under suitable conditions which allow expression of the proteins of the invention. Expression of the thermostable TX-1 xylanase enzymes of the invention may be constitutive such that they are continually produced, or inducible, requiring a stimulus to initiate expression. In the case of inducible expression, thermostable TX-1 xylanase enzyme production can be initiated when required by, for example, addition of an inducer substance to the culture medium, for example dexamethasone or IPTG.

Thermostable TX-1 xylanase enzymes of the present invention can be extracted from host cells by a variety of techniques known in the art, including enzymatic, chemical and/or osmotic lysis and physical disruption. The thermostable TX-1 xylanase enzyme is purified and isolated in a manner known *per se*.

LARGE SCALE APPLICATIONS

In one preferred embodiment, the production of the thermostable TX-1 xylanase enzyme is optimised for large scale production.

Preferably the yield of thermostable TX-1 xylanase enzyme is from about 500 to about 5000 units/kg feed.

Preferably the yield of thermostable TX-1 xylanase enzyme is from about 1000 to about 3000 units/kg feed.

The production of large yields of the thermostable TX-1 xylanase enzyme is advantageous for the preparation of food and/or feed formulations.

FOOD AND/OR FEED FORMULATION

Preferably the cereal-based food and/or feed comprises from about 100 units of thermostable TX-1 xylanase enzyme to about 2000 units of thermostable TX-1 xylanase enzyme per kilo gram (kg) of food.

Preferably the cereal-based food and/or feed comprises from about 200 units of thermostable TX-1 xylanase enzyme to about 1200 units of thermostable TX-1 xylanase enzyme per kilo gram (kg) of food.

Preferably the cereal-based food and/or feed comprises from about 300 units of thermostable TX-1 xylanase enzyme to about 600 units of thermostable TX-1 xylanase enzyme per kilo gram (kg) of food.

Preferably the cereal-based food and/or feed comprises from about 400 units of thermostable TX-1 xylanase enzyme to about 500 units of thermostable TX-1 xylanase enzyme per kilo gram (kg) of food.

Preferably the cereal-based food and/or feed comprises from about 10% to about 90% by weight of cereal.

Preferably the cereal-based food and/or feed comprises from about 20% to about 70% by weight of cereal.

Preferably the cereal-based food and/or feed comprises from about 30% to about 60% by weight of cereal.

Preferably the cereal-based food and/or feed comprises about 60% by weight of cereal.

Preferably the cereal-based food and/or feed comprises from about 10% to about 90% by weight of wheat.

Preferably the cereal-based food and/or feed comprises from about 20% to about 70% by weight of wheat.

Preferably the cereal-based food and/or feed comprises from about 30% to about 60% by weight of wheat.

Preferably the cereal-based food and/or feed comprises about 60% by weight of wheat.

In one preferred embodiment of the present invention, the cereal-based feed is prepared in a pellet form.

PRODUCTION OF PELLETS

Processing of the food and/or feed supplement into an animal feed may be performed using any of the currently used processing apparatus such as a double-pelleting machine, a steam pelleter, an expander or an extruder.

The novel thermostable TX-1 xylanase enzyme of the present invention allows the preparation of food and/or feed supplement at high temperatures without affecting the performance of the enzyme whose activity is substantially independent of any wheat inhibitors which may be present in a cereal based food and/or feed supplement. The preparation of the food and/or feed supplement of the present invention at high temperatures is highly advantageous when preparing cubes and pellets for an animal feed as these high temperatures: (i) improve the quality and durability of the resulting cubes and pellets, (ii) increase the range of ingredients which can be efficiently handled and (iii) also increase the level of liquid ingredients,such as fat and molasses, which can be incorporated into the food and/or feed.

ANIMALS

The cereal-based feed supplement of the present invention which is prepared in pellet form may be provided to animals such as turkeys, geese, ducks, sheep and cows.

Preferably the feed is provided to pigs or to poultry.

Even more preferably, the feed is provided to broiler chickens.

FOOD CONVERSION RATIO (FCR)

In one embodiment, the present invention provides a food and/or feed supplement for improving the food/feed conversion ratio (FCR) and/or increasing the digestibility of a cereal-based feed.

As used herein, the term food/feed conversion ratio (FCR) is the ratio of the amount of food and/or feed consumed relative to the weight gain of a human and/or animal subject. A low FCR indicates that a given amount of food and/or feed results in a growing human and/or animal subject gaining proportionately more weight. This means that the human and/or animal subject is able to utilise the feed more efficiently. One way in which the FCR of a food and/or feed can be improved is to increase its digestibility.

The present invention advantageously provides a food and/or feed supplement which can decrease the food and/or feed conversion ratio (FCR) of a cereal-based human food and/or animal feed.

The presence of the thermostable xylanase (TX-1) of the present invention in the resulting cereal-based food and/or feed has the advantageous and unexpected effect of reducing its FCR. That is, the thermostable xylanase (TX-1) of the present invention improves the FCR of a food and/or feed without increasing its cost per unit weight. Thus, the inclusion of the above enzyme as a feed supplement in the diet of an animal enables an animal and/or a human subject to digest the diet more efficiently. This increases the proportion of feed protein and energy which an animal and/or human subject can derive from the food and/or feed. This in turn improves the FCR of the feed making it more economical in use.

The following sample was deposited by Danisco A/S in accordance with the Budapest Treaty at the recognised depositary at the Centraalbureau voor Schimmelcultures, Patent Administration, PO Box 273, 3740 AG BAARN, The Netherlands on 2 November 1999.

Talaromyces emersonii DCDK-A65 CBS 102382

INTRODUCTION TO THE EXAMPLES SECTION AND THE FIGURES

The present invention will now be described only by way of example in which reference is made to the following Figures.

Figure 1 shows a graph;

Figure 2 shows a photographic representation;

Figure 3 shows a graph;

Figure 4 shows a graph;

Figure 5 shows a graph;

Figure 6 shows a graph;

Figure 7 shows a sequence alignment;

Figure 8a shows a sequence alignment;

Figure 8b shows a sequence alignment;

Figure 9 shows a genomic construct;

Figure 10 shows an amino acid sequence listing (SEQ ID No 1);

Figure 11 shows a nucleotide sequence listing (SEQ ID No 2);

Figure 12 shows a nucleotide sequence listing (SEQ ID No 3);

Figure 13 shows a graph;

Figure 14 shows a graph;

Figure 15 shows a graph; and

Figure 16 shows a graph.

In slightly more detail

Figure 1 shows the recovery of the thermostable *Talaromyces emersonii* (TX-1) enzyme from an ion exchange POROS 10HQ column.

Figure 2 shows a silver stained SDS polyacrylamide gel electrophoresis. Mv determination with masss spectrometry (MS) indicated two peaks with a Mv of 38,382 and 38, 987D, indicating micro heterogeneity.

Figure 3 shows the pH optimum determinations which were determined for three different xylanase preparations. These were:
TLX-1, thermostable xylanase derived from *Thermomyces lanuginosus*.
X-1 xylanase derived from *Aspergillus niger*.
TX-1 xylanase derived from *Talaromyces emersonii*.
A standard assay for optimum pH determinations was performed in citrate phosphate buffers ranging from pH 2.5 - 6.5.

Figure 4 shows the thermostability of TX-1 which was determined for three different xylanase preparations by exposing a solution of purified enzyme in acetate buffer, pH 5.0, to 40, 50, 60, 70 and 80°C for 15 minutes. After heat treatment the residual activity has been determined. The three different xylanase preparations were:
TLX-1, thermostable endo-xylanase derived from *Thermomyces lanuginosus*.
X-1 endo-xylanase derived from *Aspergillus niger*.
TX-1 endo-xylanase derived from *Talaromyces emersonii*.

Figure 5 shows the redidual xylanase activity for two enzyme preparations after subjecting the enzymes to a pelleting process. The two enzyme preparations were:
X-1 endo-xylanase derived from *Aspergillus niger* and
TX-1 endo-xylanase derived from *Talaromyces emersonii*.

Figure 6 shows the water soluble pentosan (WSP)-activity relative to activity on water insoluble pentosan (WIP) at pH 4 and pH 7. The three different xylanase preparations used were:
TLX-1, thermostable endo-xylanase from *Thermomyces lanuginosus*.
Xy-1 endo-xylanase derived from *Aspergillus niger*.
TX-1 endo-xylanase derived from *Talaromyces emersonii*.

Figure 7 shows the Primer design for degenerate PCR.

Figure 8a shows a BLAST alignment of the TX-1 xylanase amino acid sequence from *T.emersonii*, with the amino acid sequence from *A.bisporus*.

Figure 8b shows a BLAST alignment of the TX-1 xylanase amino acid sequence from *T.emersonii*, with the amino acid sequence from *H.grisea*.

Figure 9 shows the genomic organization of *tx*1 from *T. Emersonii*.

Figure 10 shows the deduced amino acid sequence for TX-1 (SEQ ID No 1).

Figure 11 shows the cDNA sequence (SEQ ID No 2) and the deduced protein sequence for TX-1.

Figure 12 shows genomic DNA (SEQ ID No 3).

Figure 13 shows the percentage relative activity of the TX-1 xylanase enzyme at pH 3-6 in the presence of a wheat extract.

Figure 14 shows the percentage relative activity of family 10 and 11 xylanase enzymes in the presence of increasing amounts of a wheat extract.

Figure 15 shows the percentage relative activity of TX-1 xylanase enzyme in the presence of xylanase inhibitor from difference cereal sources.

Figure 16 shows the temperature optimum for TX-1 enzyme determined in 0.1 acetate buffer, pH 5.0 and during a reaction time of 15 minutes.

EXAMPLES

MATERIALS AND METHODS

EXAMPLE 1

Talaromyces emersonii, CBS 841.70 was grown on CMA agar medium at 40°C to produce spores for inoculation of shake flask cultures. Shake flask medium containing ground corn cob, wheat bran and ammonium sulphate as a nitrogen source was used. Cultures were incubated at 40°C for 5-8 days at 200 rpm. Typically, 100 mg/liter of xylanase enzyme was routinely recovered.

PURIFICATION

TX-1 xylanase was purified from crude fermentation broth using successively Sephadex G25M for desalting, SOURCE 15Q anion exchanger, SOURCE 15PHE and SOURCE 15ETH for hydrophobic interaction chromatography and Superdex 75 prep grade for gel filtration.

ENZYME ASSAY

Crude culture filtrate was assayed for endo-xylanase activity with Xylazyme tablets, Megazyme, Ireland. Activity was correlated to a standard enzyme preparation and expressed as TXU/gram enzyme.

Results 1

CHARACTERISATION

(A) PURITY

The purity of the TX-1 xylanase enzyme was checked by (i) running the xylanase on MINI Q and MONO Q HR 5/5 columns (ii) SDS polyacrylamide gel electrophoresis and (iii) mass spectrometry (MS). All material used were from Pharmacia LKB Biotechnology.

The dry matter content of a purified TX-1 sample, determined by specific absorbance, was calculated to be 3.2 (0.1% at 280nm). From the specific absorbance, the specific activity was determined as 2,500 TXU/mg.

(B) SDS-PAGE

Figure 2 shows a silver stained SDS polyacrylamide gel electrophoresis. Mv determination of the staining profile of the TX-1 enzyme using masss spectrometry (MS) showed two peaks with a Mv of 38,382 and 38, 987D, indicating micro heterogeneity. The TX-1 xylanase was deemed to belong to the F family of xylanases.

(C) pH OPTIMUM

Figure 3 demonstrates the results from pH optimum determinations were carried out for three different xylanase preparations. These were:

TLX-1, thermostable endo-xylanase derived from *Thermomyces lanuginosus*.

X-1 endo-xylanase derived from *Aspergillus niger*.

TX-1 endo-xylanase derived from *Talaromyces emersonii*.

The results indicated that TX-1 is active within a rather broad pH range, pH 3-8, with the highest activity at pH 3. However, more than 50% relative activity remains at pH 6.

(D) THERMOSTABILITY

Thermostability determinations were carried out for three different xylanase preparations by exposing a solution of purified enzyme in acetate buffer, pH 5.0, to 40,

50, 60, 70 and 80°C for 15 minutes. After heat treatment the residual activity was determined. The three different xylanase preparations were:
TLX-1, thermostable endo-xylanase derived from *Thermomyces lanuginosus*.
X-1 endo-xylanase derived from *Aspergillus niger*.
TX-1 endo-xylanase derived from *Talaromyces emersonii*.

The results in Figure 4 demonstrate that, of the three enzymes studied, only TLX-1 and TX-1 are thermostable at temperatures from 60 to 80°C. TX-1 also has higher activity at these temperatures than TLX-1. The temperature optimum for TX-1 is about 80°C (see Figure 16).

(E) THERMOSTABILITY AFTER PELLETING

The residual xylanase activity for two enzyme preparations was determined after subjecting the enzymes to a pelleting process. The two enzyme preparations were:
X-1 endo-xylanase derived from *Aspergillus niger* and
TX-1 endo-xylanase derived from *Talaromyces emersonii*.

The results in Figure 5 that TX-1 maintains its activity at 90°C even after the pelleting process whereas X-1 loses its activity at this temperature and only shows a minimal activity at 80°C.

(F) WATER SOLUBLE PENTOSAN (WSP) ACTIVITY

The water soluble pentosan (WSP)-activity relative to the activity on water insoluble pentosan (WIP) was determined at pH 4 and pH 7 for three different xylanase preparations. The xylanase preparations used were:
TLX-1, thermostable endo-xylanase from *Thermomyces lanuginosus*.
Xyl-1 endo-xylanase derived from *Aspergillus niger*.
TX-1 endo-xylanase derived from *Talaromyces emersonii*.

Figure 6 demonstrates that, of the three enzymes studies, only Xyl-1 and TX-1 release WSP degradation product. A comparision of the WSP activities of Xyl-1 and TX-1 indicate that both enzymes have higher activities at pH 4 than at pH 7 but TX-1 has a higher WSP release activity than Xyl-1 at both pHs studied.

(G) XYLAN DEGRADATION PRODUCTS

Table 2 below indicates that depolymerisation of arabinoxylan with TX-1 delivers high amounts of xylobiose (X_2) and almost no xylotriose (X_3) whereas X-1 *(Aspergillus niger)* and TLX-1 *(Therinomyces lanuginosus)* deliver xylobiose (X_2) and xylotriose (X_3) in approximately the same proportions.

Table 2

	Xylose (mg/ml)	Xylobiose (mg/ml)	Xylotriose (mg/ml)
X-1	0.15	0.54	0.26
TLX-1	0.07	0.45	0.25
TX-1	0.43	1.29	0.04

(I) TYPE OF XYLANASE

Different analytical procedures for the determination of xylanase activity suggest that the TX-1 xylanase could be characterised as an exo-type xylanase but not a true exo-xylanase, where xylose is cut at the non-reducing end of arabinoxylan.

The specific activity of TX-1 compared with other known xylanases is low when procedures for measuring endo-activity are used. However, when reduced end-groups on arabinoxylan treated with TX-1 are measured, this gives a more favourable picture and the ability to reduce viscosity in a solution of water soluble pentosan is high compared with other xylanases. Thus, the TX-1 enzyme of the present invention has been classified as an endo-xylanase and not an exo-xylanase. When this xylanase is used in combination with existing xylanases; the ability to reduce viscosity of water soluble xylan is enhanced and the amount of low molecular AX-fragments is elevated in comparison with reference. The results are set out in Table 3.

Table 3: Activities of xylanases on different substrates

Substrate	arabinoxylan	arabinoxylan	arabinoxylan	arabinoxylan
Type	wheat	WIP	WSP	WSP
Origin	Megazyme	DC, Wheat	Megazyme	DC-Wheat
Assay	Xylaz.tabs	phenol-H_2SO_4	ferricyanid	Ostwald visc.
Principle	colorimetric	colorimetric	colorimetric	viscosimetric
Unit	TXU/g	WIPU/g	WSPU/g	WSPVU/g
Actual activity of enzyme preparations				
Xyl-1 (Aspergillus)	46000 (a)	46000 (a)	587	29
TX-1(Talaromyces)	419	253	109	181
TLX-1 (Thermomyces)	1317	1253	98	104
Activity/10,000 TXU				
Xyl-1	10000	10000	128	133
TX-1	10000	6038	2601	4324
TLX-1	10000	9514	744	786

a): Activity of Xyl-1 is per definition the same on Megazyme substrate and WIP-substrate
WIP-activity determined as the amount of liberated carbohydrate/gram enzyme with reference to standard enzyme
Colorimetric determination: micromol xylose/minute/gram enzyme
Viscosimetric determination:change in reciprocal specific viscosity /minute/gram enzyme
GPU-activity determined on Xylazyme tablets with reference to X-1-standard enzyme

EXAMPLE 2

Primer Design for degenerate PCR

The 5' and 3' flanking regions of the nucleotide sequence encoding the TX-1 enzyme were isolated with a genomic walking procedure as described in Siebert *et al.* 1995. This strategy resulted in the isolation of DNA fragments containing the ORF encoding TX-1.

Results 2

The results demonstrated that the ORF encoding TX-1 from *Talaromyces emersonii* (*tx*1) comprises a 1215 bp ORF interrupted by three introns of respectively 66, 54 and 45 bp in length which encode a protein of 405 amino acids. The protein sequence was

completely identical to the protein sequence of the fragments obtained by protein sequencing.

Sequence Identity

This protein had less than 55% identity to xylanases from other organisms such as *A.bisporus* 52% identical aa (see Figure 8a) and *H. grisea* 41% identical aa (see Figure 8b).

Sequence Characterisation

The TX-1 protein comprises a predicted signal sequence which is cleaved between aa 23-24 leaving a mature protein of 382 aa (deduced MW 40.8 kDa). A glycosyl hydrolase F10 active site is located at aa 254-264 and a putative cellulose binding domain can be found at the extreme C-terminus of the protein. TX-1 contains also two putative *N*-linked glycosylation sites. These sites are illustrated in the schematic diagram in Figure 9. The active enzyme comprises a 330 amino acid sequence.

EXAMPLE 3

The percentage relative activity of the thermostable TX-1 xylanase enzyme of the present invention was assayed in the presence of a wheat derivable extract (200ul) at pH in the range of 3-6.

Results 3

The results shown in Figure 13 indicate that the activity of the thermostable TX-1 xylanase enzyme of the present invention is substantially the same at pH in the range of 3-6 but with the highest activity at pH 3.0.

EXAMPLE 4

Influence of wheat derived xylanase inhibitor on xylanase activity.

The influence of a wheat derivable xylanase inhibitor was investigated for four different xylanase preparations from family 10 and family 11 xylanase families.

X-3, xylanase from *Aspergillus niger* (family 10 xylanase)
NN, xylanase (Biofeed Wheat) from Novo Nordisk (family 11 xylanase)
X-1 xylanase derived from *Aspergillus niger* (family 11 xylanase)
TX-1 xylanase derived from *Talaromyces emersonii* (family 10 xylanase)

3 gram non-pelleted food and/or feed was extracted with 30 ml 0.1 M citrate phosphate buffer (CPB), pH 6.50 or 30 ml 0.1 M acetate buffer, pH 5.00 for 30 minutes and filtered. 4 mixtures were prepared:

1,000 µl buffer + 0 µl extract.
900 µl buffer + 100 µl extract.
800 µl buffer + 200 µl extract.
600 µl buffer + 400 µl extract.

Each mixture is incubated at 40°C with 100 µl enzyme solution for 10 minutes and 10 ml 2% TRIS stop-solution is added. Samples are filtered and ΔOD measured at 590 nm.

Results 4

Figure 14 shows that the percentage relative activity of four different xylanase enzymes when assayed in the present of 1, 100, 200 and 400 ul of a wheat extract. Two of the four enzymes studied (X-3, NN) showed at least a 50% reduction in activity after addition of 100ul of the wheat extract. The X-1 enzyme showed at least a 50% reduction in activity after addition of 200 ul of the wheat extract. In contrast, the TX-1 xylanase enzyme of the present invention showed less than a 10% reduction in activity

after addition of either 100ul or 200ul of the wheat extract. Thus these results demonstrate that the activity of the thermostable TX-1 xylanase enzyme of the present invention is substantially independent of wheat derivable xylanase inhibitors.

EXAMPLE 5

Food and/or feeding Trial

The main objective of this food and/or feeding trial was to study the efficacy of two enzyme preparations (Grindazym GP 5000 (Reference Enzyme) and Test product TS-E 389) in a 60% wheat diet for broilers.

MATERIALS AND METHODS

(A) ANIMALS, HOUSING AND MANAGEMENT

The experiment was conducted in the experimental farm of the Centre de Mas Bov6, of IRTA, Ctra. Reus a El Morell, kin. 4.5, 43120 Constanti (Spain).

Two hundred and eighty eight male broiler chicks of the Ross strain were used. The chicks were allocated to 48 departments located in two Petersime (Petersime Incubator Company, Gettysburg, Ohio, USA) battery brooders, provided with electrical heating. Each replicate consisted of 6 males. The departments were 0.376 m^2 each, so that stocking density was 16 birdS/m^2. The batteries are located in a room provided with forced ventilation. The experiment lasted 25 days (from 20/1/97 to 13/I/97). Light and temperature program were the standard used in the farm. The temperatures and the light were adjusted according to the following program:

Ist week: 30-35°C	0-4 days: 23 h of light
2nd week: 29-32°	4-10 days: 20 h of light
3rd week: 27-30°C	10 days to the end: 18 h of light

Typical prophylactic cleaning, disinfecting and vaccination were carried out according to the routine practice. Food and/or feed and water were provided *ad-libitum* throughout the experiment. Birds were vaccinated against infectious bronchitis at the hatchery.

(B) TREATMENTS AND EXPERIMENTAL DESIGN

There were six experimental treatments replicated eighth times and allocated at random by blocks, according to the location in the experimental room. Each replicate contained six males. The six experimental treatments were set out in the Table 5 below.

Table 5

Treatments	**Diet**	**Enzyme Preparation**	**Dose TXU/kg**	**Dose**
T-1		No	-	
T-2		Grindazym GP 5000 reference enzyme	6,000	500 ppm
T-3	Basal diet	TS-E 389 Test enzyme	2,000	Level 1
T-4	60% wheat	Test enzyme	1,200	60% of Level 1
T-5		Test enzyme	720	35% of Level 1
T-6		Test enzyme	430	21.6% of Level 1

(C) FOOD AND/OR FEED MANUFACTURE AND NUTRITIONAL COMPOSITION OF THE DIETS

Mash fees were used in this trial. Food and/or feeds were mixed at the food and/or feedmill belonging to the Department of Animal Nutrition of IRTA in Mas Bové. A single diet was offered throughout the experiment. Five hundred kg of the basal diet was prepared in an horixontal mixer of 500 kg capacity. An eighty kg aliquot of the diet was taken for each treatment, and the enzyme preparation was premixed with 800 g of wheat flour and subsequently mixed with the rest of the food and/or feed in a concrete

mixer. The time of mixing was 5 min. For the negative control diet (T-1) a control blank premix (800 g) was added . Enzyme premixes were prepared by Danisco Ingredients. The composition of the diet is shown in Table 6.

Table 6: Composition of the experimental diets

Ingredient	%
Wheat, *Cartaya*	60.0
Animal and vegetable fat	4.0
Full fat extruded soybeans	11.6
Soybean meal	20.3
DL-methionine	0.190
L-lysine HCl	0.160
Calcium carbonate	1.250
Dicalcium phosphate	1.770
Salt	0.330
Minerals and vitamins[1]	0.400

The wheat used was of the *Cartaya* type, harvested in the Lleida area and its analytical composition is shown in Table 7.

Table 7 – Analytical composition of *Cartaya* wheat (on dry matter basis)

Parameter	%
Specific gravity (kg/HL	75.9
Dry matter	88.7
Crude protein	13.7
Ether extract	2.04
Ash	1.96
Crude fiber	2.88
Total β-glucans	0.49

Insoluble β-glucans	0.47
Total pentosans	9.98
Soluble pentosans	1.33

(D) No other food and/or feed activities were used in the trial

(E) CONTROLS

Feeds and wheat sample: before analysis, all samples of experimental diets and *Cartaya* wheat were ground through 1.0 mm screen on a cyclone sample mill. The analytical composition of *Cartaya* wheat was determined, including specific gravity, dry matter, crude protein, crude fat, ash, crude fiber, total and insoluble β-glucans, total and soluable pentosans. Quality control of the manufactured food and/or feed was performed determining dry matter, crude protein, ether extract, crude fiber and ash (Table 8).

Table 8. Analytical composition of experimental diets (% on air basis)

Diet	Dry Matter	Crude protein	Ether extract	Crude fiber	Ash
T-1	11.43	10.17	7.42	3.34	5.44
T-2	11.25	20.42	7.28	3.34	5.69
T-3	11.17	20.58	7.48	3.20	5.66
T-4	11.24	20.37	7.43	3.35	5.37
T-5	11.02	20.45	7.62	3.38	5.55
T-6	11.06	20.3	7.59	3.34	5.45

Two kg of samples of the feeds and one kg of *Cartaya* wheat were sent to Danisco Ingredients for enzyme activity analysis.

Chicks were bulk-weighed on arrival at the farm. Feed and chicks were weighed on day 21, and feed consumption, average daily gain and feed efficiency were calculated.

Mortality was checked and recorded daily, including the cause of death. Percentage of animals with sticky droppings at 12 days; Litter condition at 19 days; Dry matter content of excreta at 16 days; Water intake, food and/or feed intake and water to food and/or feed ratio from 15 to 17 days.

At 18 days, experimental diets (T-1 and T-2) were offered containing 0.3% of Cr_2O_3 as a tracer. At 24 and 25 days, birds were killed by intravenous injection of sodium pentobarbital and chicks and their pancreas were weighed. Digesta samples from Meckel's diverticulum to 15 cm before ileo-cecal conjunction were taken and stored on ice to measure fresh digesta viscosity. The chyme samples were pooled per intestinal segment per cage (eight samples from two animals per treatments). Moreover, samples from the last part of ileum (I 5 cm) from treatments T- I and T-2 were taken, frozen and freeze-dried to measure ileal amino acid digestibility (eight samples from six animals per treatment).

To measure the viscosity, samples from fresh digesta were centrifuged at 12000 rpm for 5 min. at 15'C. The supernatant was collected and stored in ice. Viscosity was determined using a Brookfield digital viscometer (model LVTDVCP-11, Brookfield Engineefing Laboratories, Stoughton, MA) maintained at 30'C and reading after I min.

To determine ileal amino acid digestibility, the chromium oxide content of diets and faeces was determined by a spectrophotometric method after alkaline fusion (Aguilera *et al.*, 1988). Feed and digesta amino acid content were measured in a Beckman System Gold apparatus, using ion exchange separation and post column derivatization with ninhydrin .

(F) STATISTICAL ANALYSIS

Firstly, complete date were analyzed as a randomized complete block design, with a twoway variance analysis, with eight blocks and six treatments. To compare means between T-1 (without enzyme) and the five treatments with enzyme addition, the Dunnett's test procedure was applied. Dunnett's procedure compares the control with the average of all the other treatments. Secondly, to compare the different treatments with enzyme addition, data without the treatment T-1 (control) were analyzed as a randomized complete block design, with a two-way analysis of variance, with blocks and treatments. Treatments means were separated by a Duncan multiple range test. Thirdly, data from treatments T-1, T-3, T-4, T-5 and T-6 were subjected to a linear and quadratic regression analysis. In the ANOVA, the sum of squares (s.s.) for diet was partitioned into a linear and quadratic relationship with Y to analyze differences in the magnitude of the effect of increasing dose of TS-E 389 enzyme. When the quadratic term was statistically significant but its s.s. was only a small part of the treatments s.s., it was fitted a linear trend only.

Data from pancreas weight was analyzed as a single-factor experiment in randomized blocks with sub sampling and pen was taken as experimental unit. Analysis of percentage data were performed with arc sine of the square root transformations, and viscosity data with log transformations to homogenize the variance.

(H) UNFAVORABLE EFFECTS AND OTHER INCIDENTS

Any unfavorable effect or other incidents were recorded.

Results 5

Performance between 0 and 21 days is shown in Table 9.

Table 9 Performance of birds from 0 to 21 days

Treatment	Enzyme	Dose	Body weight (g)	Body weight gain (g/day)	Food and/or feed intake (g/day)	Food and/or feed Efficiency (FCR)
T-1	No		695	31.1	49.1	**1.578a**
T-2	ref.enz.	500 ppm	698	31.3	48.5	**1.554a**
T-3	TX-1	Level 100	702	31.5	46.4	**1.475b**
T-4	TX-1	Level 60	707	31.7	46.3	**1.459b**
T-5	TX-1	Level 36	715	32.1	47.7	**1.484b**
T-6	TX-1	Level 21.6	717	32.2	47.8	**1.487b**
SE			13.1	0.62	0.94	0.0154
Significance of dose for test enz. in linear model			0.168	0.172	0.825	**0.018**
Singnificance of dose for test enz. in quadratic model			0.405	0.382	*0.088*	**0.0001**
Significance of dose^2 for test enz. in quadratic model			0.650	0.616	*0.087*	**0.0001**

Values are means of eight replicates of six chicks.

Statistical analysis (Dunnett's test) shown significant differences ($P<0.05$) in body weight at 21 days and daily weight gain between treatments T- I and T-3 and between T- I and T-6. No significant differences between control diet and diets with enzyme addition were observed on food and/or feed efficiency and daily food and/or feed intake. The mortality in the overall period was 1.74%.

No significant differences on performance parameters between the five treatments with enzyme addition were observed. Regression analysis showed a significant and positive linear effect (Table 5) of the dose of TS-E 389 enzyme on food and/or feed efficiency ($P<0.01$).

SUMMARY

The inclusion of a thermostable xylanase in an animal food and/or feed in accordance with the present invention enables the crude protein value and/or digestibility and/or the amino acid content and/or digestibility coefficients of the food and/or feed to be increased, which permits a reduction in the amounts of alternative protein sources and/or amino acids supplements which have previously had to be included in animal food and/or feeds. When the protein digestibility coefficient and/or the content of available crude protein of wheat is increased by the addition of the thermostable TX-1 xylanase of the present invention, major savings can be found in the reduced levels of protein and/or energy supplements which have conventionally needed to be added.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

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Tuohy M G, Puls J, Claeyssens M, Vrsanska M, & Coughlan, M P (1993a). The xylan-degrading enzyme system of *Talaromyces emersonii*; novel enzyms with activity against aryl β-D-xylosides and unsbustituted xylans, *Biochem. J. 290,* 515-23.

Tuohy M G & Coughlan M P (1992). Production of terhmostable xylan-degrading enzymes by *Talaromyces emersonii. Biores. Technol.,* **39,** 131-7.

Tuohy M G, Coughlan T L & Coughlan M P (1990) Solid-state versus liquid cultivation of *Talaromyces emersonii* on straws and pulps: enzyme productivity. In *Advances in Biological Treatment of Lignocellulosie Materials.* Ed. M P Coughlan & M T Amaral-Collaco Elsevier Applied Science, London pp 153-75.

Voragen. A G J, Gruppen, H, Verbruggen, M A & Victor R J, (1992). Characterization of cereal arabinoxylans. In *Xylans and Xylanases.* Ed J Visser, G Beldman, M A Kusters-van Someren & A G J Voragen. Elsevier Amsterdam, pp 51-67.

Wong K K Y Tan L U L & Saddler, J N (1988). Multiplicity of β-1,4-xylanase in microorganisms: functions and applications. *Microbiol. Rev, 52,* 305-17.

57

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 34, line 15-20

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution

CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution *(including postal code and country)*

PATENT ADMINISTRATION
PO BOX 273
3740 AG BAARN
THE NETHERLANDS

Date of deposit	Accession Number
2 NOVEMBER 1999	CBS 102382

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will only be made available either until the publication of the mention of the grant of the patent or after twenty years from the date of filing if the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☑ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer A. Grace	Authorized officer

CLAIMS

1. A thermostable xylanase enzyme capable of modifying a xylan polymer in a food and/or feed supplement wherein the activity of the xylanase enzyme is substantially independent of any level of a wheat xylanase inhibitor that may be present in the food and/or feed supplement.

2. The xylanase enzyme according to claim 1 wherein the xylanase enzyme is obtainable from *T. emersonii.*

3. The xylanase enzyme according to claim 1 or claim 2 wherein the xylanase enzyme is obtainable from *T. emersonni* and wherein the enzyme has the following characteristics:

(i) a MW of about 38, 000 Daltons;

(ii) arabinoxylan degrading activity;

(iii) water soluble pentosan (WSP) activity;

(iv) a pH optima of from about 3.0 to about 8.0 (more especially from about 2.3 to about 3.6, more especially about 3.0);

(v) a temperature optima of from about 65°C to about 95°C (more especially from about 75°C to about 85°C), more especially about 80°C; and

(vi) retention of arabinoxylan degrading activity after exposure to wheat xylanase inhibitors

4. The xylanase enzyme according to claim 1 or claim 2 or claim 3 wherein the xylanase enzyme is prepared by recombinant DNA techniques.

5. The xylanase enzyme according to any of the preceding claims wherein the xylanase enzyme comprises the amino acid sequence presented as SEQ ID No 1 or a variant, homologue, fragment or derivative thereof.

6. A nucleotide sequence encoding the xylanase enzyme according to any one of claims 1-5.

7. A nucleotide sequence according to claim 6 comprising the sequence presented as SEQ ID No. 2 or a variant, homologue, fragment or derivative thereof.

8. A nucleotide sequence capable of hybridising to the nucleotide sequence according to claim 6 or claim 7 or a sequence that is complementary to the hybridisable nucleotide sequence.

9 A nucleotide sequence according to claim 6 or claim 7 or claim 8 wherein the nucleotide sequence is operably linked to a promoter.

10. A construct comprising the nucleotide sequence according to any one of claims 6-9.

11. A vector comprising the nucleotide sequence of any one of claims 6-10.

12. A plasmid comprising the nucleotide sequence of any one of claims 6-11.

13. A host cell comprising the nucleotide sequence of any one of claims 6-12.

14. A process for preparing a xylanase enzyme according to any one of claims 1-5 comprising expressing a nucleotide sequence according to any one of claims 6-9 or when present in the invention of any one of claims 10-13 and optionally isolating and/or purifying the enzyme.

15. A xylanase enzyme produced by the process according to claim 14.

16. A method for modifying a xylan in a food supplement comprising: providing a food supplement comprising a xylan; contacting the food and or feed supplement with a xylanase enzyme according to any one of claims 1-5 or as defined in claims 14-15; and

causing the xylan polymer to be modified by the xylanase enzyme under conditions sufficient for its modification.

17. The method according to claim 1 or any dependent claim theron or claim 16 wherein the xylan polymer is derivable from a cereal.

18. The method according to claim 16 or claim 17 wherein the cereal is a wheat cereal.

19. A food and/or feed supplement comprising a modified xylan produced by the method of claim 16 or claim 17 or claim 18.

20. A food and/or feed supplement according to claim 18 wherein the food and/or feed supplement is a added to an animal feed or a food.

21. A method of improving the food and/or feed conversion ratio (FCR) and/or increasing the digestibility of a cereal based food and/or feed in a human and/or animal subject, the method comprising introducing into the subject a food and/or feed supplement comprising a modifed xylan as defined in claim 1 or any dependent claim thereon or as defined in claim 19.

22. The method according to claim 21 whereby the food and/or feed supplement comprises a xylanase enzyme according to any one of claims 1-5 or claim 15 or as defined in claim 14 in an amount that the feed comprises 200-2000 units of xylanase enzyme activity per kg.

23. The method according to claim 21 or claim 22 wherein the food and/or feed supplement comprises at least 60% by weight of wheat (and/or maize).

24. A xylanase enzyme capable of modifying a xylan polymer in a food and/or feed supplement wherein the xylanase enzyme is contacted with the xylan polymer under conditions comprising:

(i) a temperature from about 65-95°C (more especially from about 75 to 85°C, more especially about 80°C;

(ii) the presence of naturally occuring cereal xylanase inhibitors (more especially a wheat xylanase inhibitors); such that a substantially degraded xylan polymer is obtained.

25. The use of a xylanase enzyme according to any one of claims 1-5 or claim 15 or claim 24 or as defined in claim 14 or claim 22 to degrade a xylan polymer in a food and/or feed supplement.

26. The use of a xylanase enzyme according to any one of claims 1-5 or claim 15 or claim 24 or as defined in claim 14 or claim 22 or claim 25 to prepare a food and/or feed supplement.

27. The use according to claims 25 or claim 26 wherein the xylanase enzyme is used in combination with another enzyme, preferably an enzyme selected from the group consisting of a cellulase, a hemicellulase, a xylanase, a pentosanase, an amylase, a lipase and a protease.

28. The use of a food and/or feed supplement according to claim 19 or claim 20 or as defined in any one of claims 1, 16, 22-24 wherein the food and/or feed products are capable of improving the food and/or feed conversion ratio (FCR) and/or increasing the digestibility of a cereal based food and/or feed in a human and/or animal subject,

29. The use of a xylanase enzyme according to any one of claims 1-5 or claim 15 or claim 24 or prepared by the method according to claim 15 in the manufacture of a medicament or foodstuff to reduce or present indigestion and/or increase nutrient absorption and/or to affect intestinal microflora development.

30. A composition comprising a food and/or feed supplement according to claim 19 or claim 20 or as defined in any one of claims 1, 16, 22-24 and at least one additional food component.

31. A food and/or feed supplement according to claim 19 or claim 20 or as defined in any one of claims 1, 16, 22-24 or claim 28 for improving the food and/or feed conversion ratio (FCR) and/or increasing the digestibility of a cereal-based food and/or feed.

32. An enzyme and a method substantially as described herein and with reference to the accompanying Figures.

33. An enzyme according to the present invention or a nucleotide sequence encoding same, obtainable from DCDK-A65 CBS 102382.

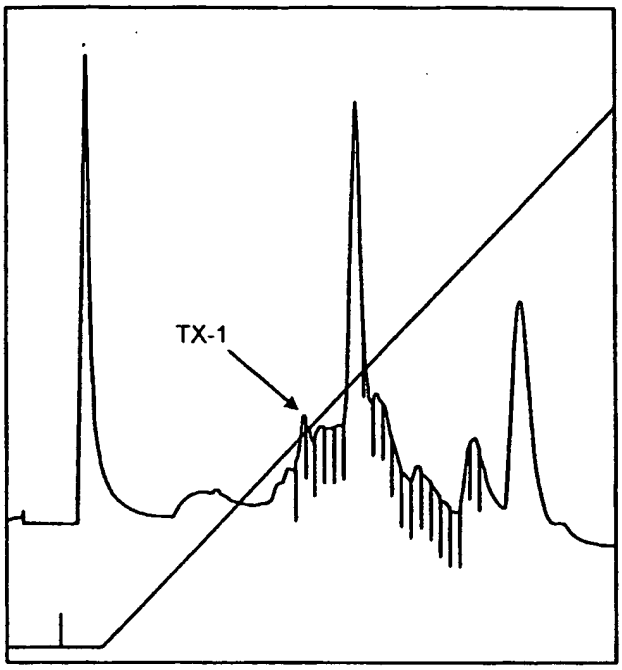

TX-1

FIG. 1

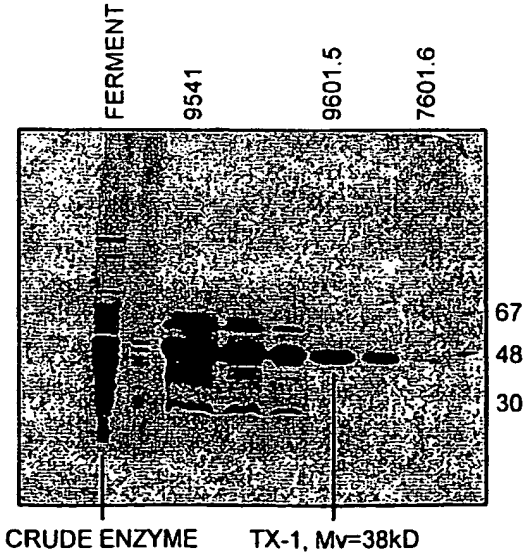

FERMENT
9541
9601.5
7601.6
67
48
30
CRUDE ENZYME
TX-1, Mv=38kD

FIG. 2

FIG. 3

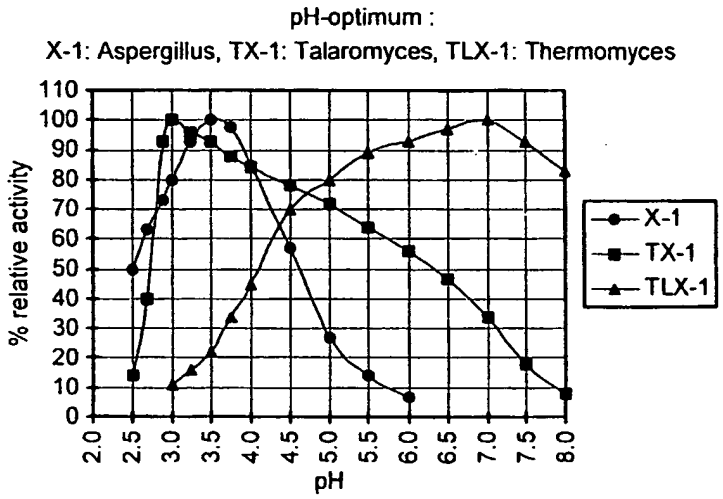

pH-optimum :
X-1: Aspergillus, TX-1: Talaromyces, TLX-1: Thermomyces
% relative activity
110
100
90
80
70
60
50
40
30
20
10
0
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
pH
X-1
TX-1
TLX-1

FIG. 4

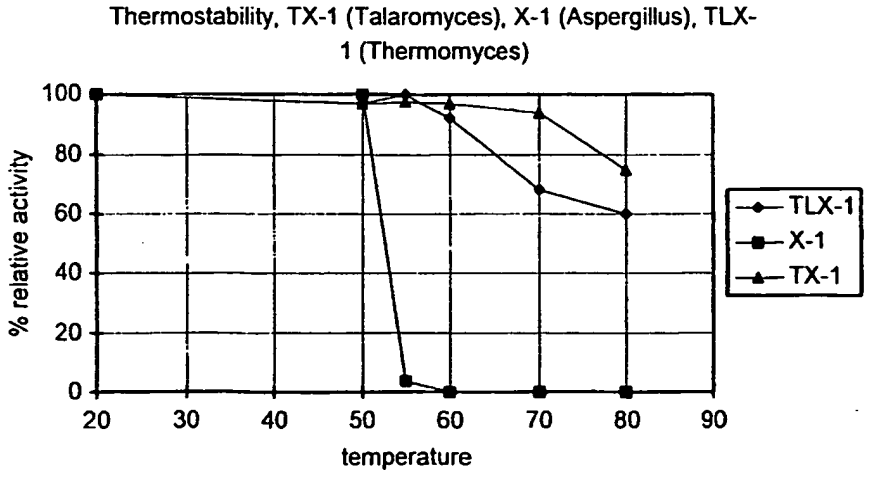

Thermostability, TX-1 (Talaromyces), X-1 (Aspergillus), TLX-1 (Thermomyces)
% relative activity
100
80
60
40
20
0
20
30
40
50
60
70
80
90
temperature
TLX-1
X-1
TX-1

FIG. 5

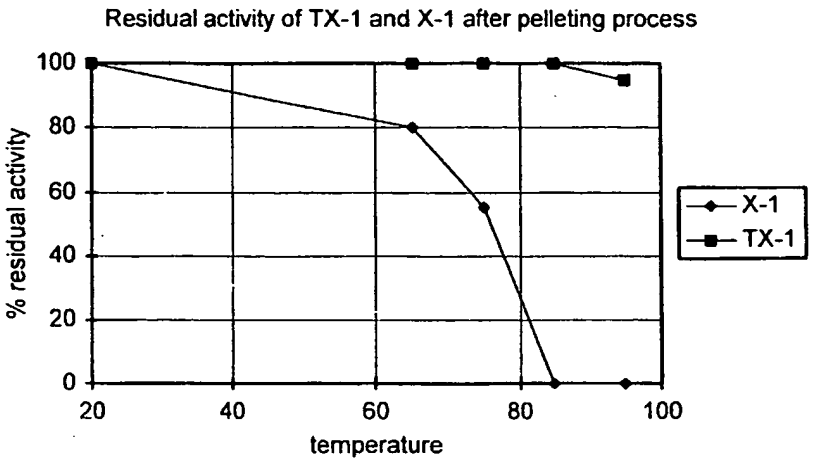

Residual activity of TX-1 and X-1 after pelleting process
% residual activity
100
80
60
40
20
0
20
40
60
80
100
temperature
X-1
TX-1

FIG. 6

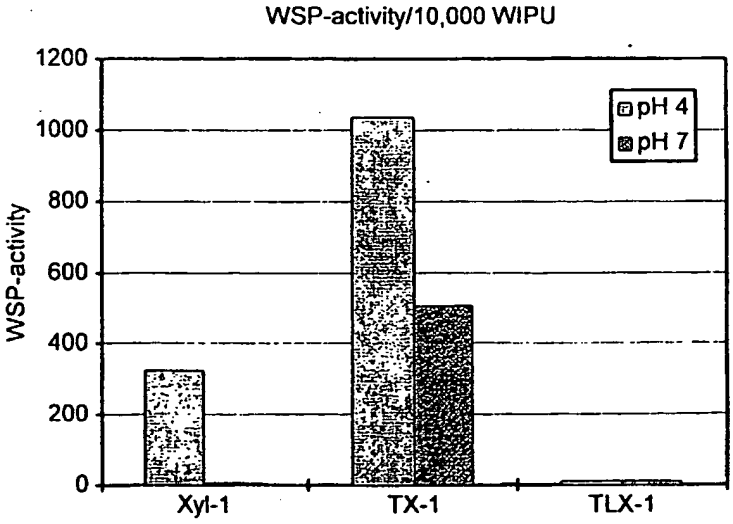

WSP-activity/10,000 WIPU
WSP-activity
1200
1000
800
600
400
200
0
pH 4
pH 7
Xyl-1
TX-1
TLX-1

FIG. 7

PRIMER design for degenerate PCR

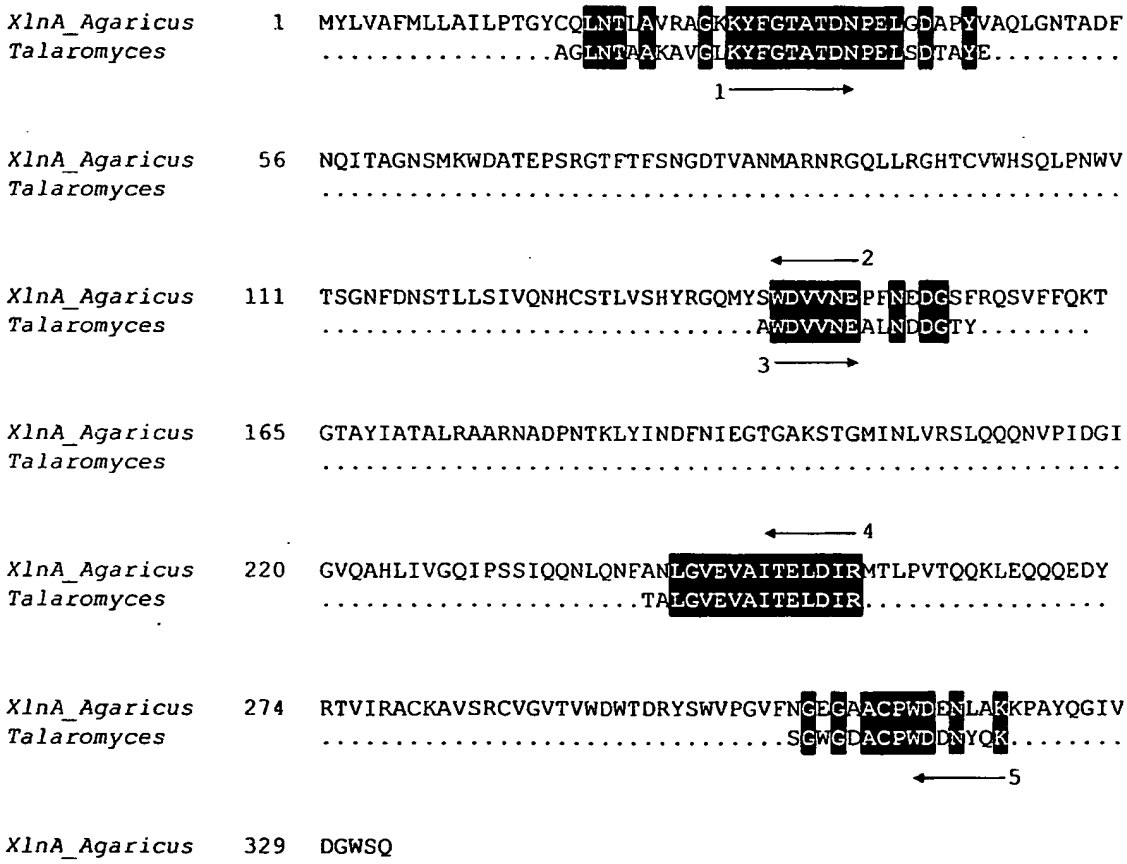


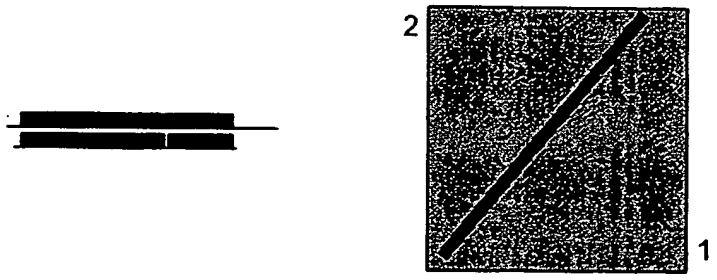
FIG. 8A

NCBI *Entrez* *BLAST 2 sequences* *BLAST* *Help*

BLAST 2 SEQUENCES RESULTS VERSION BLASTP 2.0.9 [May-07-1999]

Matrix: 0 BLOSUM gap open: 11 gap extension: 1
x_dropoff: 50 expect: 10.00 wordsize: 3 Filter: X Align

Sequence 1 lcl|seq_1 **Length** 404(1 .. 404)
Sequence 2 lcl|seq_2 **Length** 333(1 .. 333)



NOTE:The statistics (bitscore and expect value) is calculated based on the size of nr database

Score = 354 bits (898), Expect = 6e-97
Identities = 164/312 (52%), Positives = 209/312 (66%), Gaps = 2/312 (0%)

To Agaricus Bisporus Xylanase

AGARICUS BISPORU

```
Query:26  LNTAAKAVGLKYFGTATDNPELSDTAYETELNNTQDFGQLTPANSMKWDATEPQQNTFTF 85
          LNT A   G KYFGTATDNPEL D  Y  +L NT DF Q+T  NSMKWDATEP + TFTF
Sbjct:19  LNTLAVRAGKKYFGTATDNPELGDAPYVAQLGNTADFNQITAGNSMKWDATEPSRGTFTF 78

Query:86  SGGDQIANLAKANGQMLRCHNLVWYNQLPSWVTGGSWTNETLLAAMKNHITNVVTHYKGQ 145
          S GD +AN+A+  GQ+LR H  VW++QLP+WVT G++ N TLL+ ++NH + +V+HY+GQ
Sbjct:79  SNGDTVANMARNRGQLLRGHTCVWHSQLPNWVTSGNFDNSTLLSIVQNHCSTLVSHYRGQ 138

Query:146 CYAWDVVNEALNDDGTYRSNVFYQYIGEATSPIAFATPPPPTPTPSCTTTTTTSSTPGVK 205
           Y+WDVVNE  N+DG++R +VF+Q  G A    A        P               G K
Sbjct:139 MYSWDVVNEPFNEDGSFRQSVFFQKTGTAYIATALRAARNADPNTKLYINDFNIEGTGAK 198

Query:206 ATAAQNIVKLVQSYGARIDGVGLQSHFIVGQTPSTSAQQQNMAAFTALGVEVAITELDIR 265
          +T   N+V+ +Q     IDG+G+Q+H IVGQ PS+   QQN+  F  LGVEVAITELDIR
Sbjct:199 STGMINLVRSLQQQNVPIDGIGVQAHLIVGQIPSSI--QQNLQNFANLGVEVAITELDIR 256

Query:266 MQLPETSAQLTQQATDYQSTVQACVNTDSCVGITLWDWTDKYSWVPSTFSGWGDACPWDD 325
          M LP T  +L QQ  DY++ ++AC     CVG+T+WDWTD+YSWVP  F+G G ACPWD+
Sbjct:257 MTLPVTQQKLEQQQEDYRTVIRACKAVSRCVGVTVWDWTDRYSWVPGVFNGEGAACPWDE 316

Query:326 NYQKKPAYNGIL 337
          N  KKPAY GI+
Sbjct:317 NLAKKPAYQGIV 328

CPU time:    0.07 user secs.        0.04 sys. secs        0.11 total secs.

Gapped
Lambda     K        H
   0.270    0.0470    0.230
```

FIG. 8A CONT'D

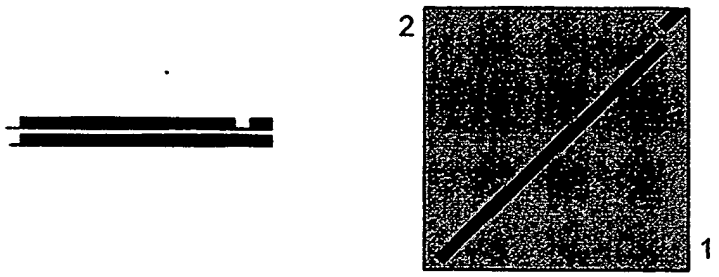
FIG. 8B

NCBI *Entrez* *BLAST 2 sequences* *BLAST* *Help*

BLAST 2 SEQUENCES RESULTS VERSION BLASTP 2.0.9 [May-07-1999]

Matrix: 0 BLOSUM gap open: 11 gap extension: 1
x_dropoff: 50 expect: 10.0000 wordsize: 3 Filter: X Align

Sequence 1 lcl|seq_1 **Length** 404(1 .. 404)
Sequence 2 lcl|seq_2 **Length** 429(1 .. 429)



NOTE:The statistics (bitscore and expect value) is calculated based on the size of nr database

Score = 332 bits (843), Expect = 2e-90
Identities = 173/413 (41%), Positives = 221/413 (52%), Gaps = 32/413 (7%)

To Humicola Grisea Xylanase

HUMICOLA GRISEA

```
Query:23   AAGLNTAAKAVGLKYFGTATDNPEL-SDTAYETELNNTQDFGQLTPANSMKWDATEPQQN 81
           +A L+  A   GLKYFGTA      + SD  Y   L++T++FGQL P N  KWDATEP +
Sbjct:18   SAQLHELAVKAGLKYFGTALREGAINSDQQYNRILSDTREFGQLVPENGQKWDATEPNRG 77

Query:82   TFTFSGGDQIANLAKANGQMLRCHNLVWYNQLPSWVTGGSWTNETLLAAMKNHITNVVTH 141
            F F  GD  AN A+ NGQ LRCH L+WY+QLP WV+ G+W  +TL A MK HI NV+ H
Sbjct:78   QFNFQQGDITANKARQNGQGLRCHTLIWYSQLPGWVSSGNWNRQTLEAVMKTHIDNVMGH 137

Query:142  YKGQCYAWDVVNEALNDDGTYRSNVFYQYIGEATSPIAFATPPPPTPTPSCTTTTTTSST 201
           YKGQCYAWDVVNEA++D+G +R+NVF +  G    P++F       P
Sbjct:138  YKGQCYAWDVVNEAVDDNGQWRNNVFLRVFGTDYLPLSFNLAKAADPDTKLYYNDYNLEY 197

Query:202  XGVKATAAQNIVKLVQSYGARIDGVGLQSHFIVGQTPSTSAQQQNMAAFTALGVEVAITE 261
             K   A  +VK+VQ  GA IDGVG Q H IVG TP+       +  FT+LGVEVA TE
Sbjct:198  NQAKTDRAVELVKIVQDAGAPIDGVGFQGHLIVGSTPTRQQLATVLRRFTSLGVEVAYTE 257

Query:262  LDIR-MQLPETSAQLTQQATDYQSTVQACVNTDSCVGITLWDWTDKYSWVPSTFSGWGDA 320
           LDIR  +LP +   L  Q  D+ + V +C++   CVG+T+W +TDKYSW+P TFSG GDA
Sbjct:258  LDIRHSRLPASQQALVTQGNDFANVVGSCLDVAGCVGVTVWSFTDKYSWIPETFSGEGDA 317

Query:321  CPWDDNYQKKPAYNGILTALGGT----PSSSTSYTLTPTTT---------------------- 368
            +D N+ KKPA+  I + L        P+SS++  + PTTT
Sbjct:318  LIYDRNFNKKPAWTSISSVLAAAAATNPPASSSTSVVVPTTTFVTTTTTPPPISSPIVPST 377

Query:369  ------------------AQHWEQCGGLGLGLGPTVCASGFTCTVINEYYSQCL 404
                              W QCGG+G   GPT C S +TCT +N++Y QCL
Sbjct:378  TTTSAVPTTTVSPPEPEQTRWGQCGGIGWN-GPTKCQSPWTCTRLNDWYFQCL 429

CPU time:     0.11 user secs.      0.02 sys. secs          0.13 total secs.

Gapped
```

FIG. 8B CONT'D

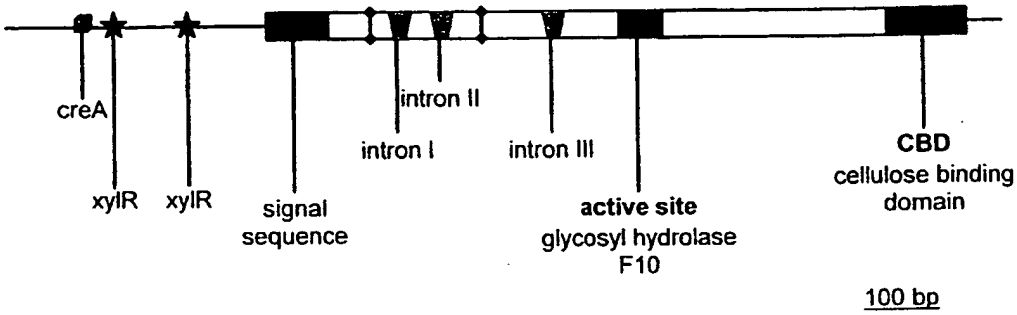


FIG. 9

FIG. 10

```
                5         10        15        20        25        30
                |         |         |         |         |         |
  1 M V R L S P V L L A S I A G S G L P L Y A Q A A G L N T A A
 31 K A V G L K Y F G T A T D N P E L S D T A Y E T E L N N T Q
 61 D F G Q L T P A N S M K W D A T E P Q Q N T F T F S G G D Q
 91 I A N L A K A N G Q M L R C H N L V W Y N Q L P S W V T G G
121 S W T N E T L L A A M K N H I T N V V T H Y K G Q C Y A W D
151 V V N E A L N D D G T Y R S N V F Y Q Y I G E A T S P I A F
181 A T P P P P T P T P S C T T T T T T S S T P G V K A T A A Q
211 N I V K L V Q S Y G A R I D G V G L Q S H F I V G Q T P S T
241 S A Q Q Q N M A A F T A L G V E V A I T E L D I R M Q L P E
271 T S A Q L T Q Q A T D Y Q S T V Q A C V N T D S C V G I T L
301 W D W T D K Y S W V P S T F S G W G D A C P W D D N Y Q K K
331 P A Y N G I L T A L G G T P S S S T S Y T L T P T T T P S G
361 G S G S P T D V A Q H W E Q C G G L G L G L G P T V C A S G
391 F T C T V I N E Y Y S Q C L
```

FIG. 11

```
        10         20         30         40         50         60
         |          |          |          |          |          |
ATGGTTCGCCTCAGTCCAGTCCTGCTGGCATCGATCGCAGGCTCTGGCCTGCCTCTGTAC
 M  V  R  L  S  P  V  L  L  A  S  I  A  G  S  G  L  P  L  Y

        70         80         90        100        110        120
         |          |          |          |          |          |
GCACAAGCAGCCGGCCTCAACACCGCCGCCAAAGCCGTCGGCCTGAAATACTTCGGCACG
 A  Q  A  A  G  L  N  T  A  A  K  A  V  G  L  K  Y  F  G  T

       130        140        150        160        170        180
         |          |          |          |          |          |
GCGACCGACAACCCCGAACTGAGCGACACCGCGTACGAGACGGAGCTGAACAACACGCAG
 A  T  D  N  P  E  L  S  D  T  A  Y  E  T  E  L  N  N  T  Q

       190        200        210        220        230        240
         |          |          |          |          |          |
GATTTCGGGCAGTTGACACCTGCGAATTCGATGAAATGGGACGCAACCGAGCCCCAGCAA
 D  F  G  Q  L  T  P  A  N  S  M  K  W  D  A  T  E  P  Q  Q

       250        260        270        280        290        300
         |          |          |          |          |          |
AACACTTTCACGTTCAGCGGCGGCGATCAGATCGCTAACCTGGCCAAGGCGAATGGCCAG
 N  T  F  T  F  S  G  G  D  Q  I  A  N  L  A  K  A  N  G  Q

       310        320        330        340        350        360
         |          |          |          |          |          |
ATGTTGAGGTGCCATAATCTTGTTTGGTATAATCAGTTGCCGTCGTGGGTCACCGGTGGA
 M  L  R  C  H  N  L  V  W  Y  N  Q  L  P  S  W  V  T  G  G

       370        380        390        400        410        420
         |          |          |          |          |          |
TCCTGGACCAACGAGACGCTGCTTGCTGCCATGAAGAATCACATCACAAACGTCGTTACC
 S  W  T  N  E  T  L  L  A  A  M  K  N  H  I  T  N  V  V  T
```

```
      430       440       450       460       470       480
       |         |         |         |         |         |
CATTACAAGGGCCAGTGCTATGCATGGGATGTCGTGAATGAGGCCCTCAACGACGACGGC
 H  Y  K  G  Q  C  Y  A  W  D  V  V  N  E  A  L  N  D  D  G

      490       500       510       520       530       540
       |         |         |         |         |         |
ACCTACCGCAGCAACGTCTTCTACCAGTATATCGGGGAGGCTACATCCCCCATCGCCTTC
 T  Y  R  S  N  V  F  Y  Q  Y  I  G  E  A  T  S  P  I  A  F

      550       560       570       580       590       600
       |         |         |         |         |         |
GCGACGCCGCCGCCGCCGACCCCGACGCCAAGCTGTACTACAACGACTACAACATCGAGT
 A  T  P  P  P  P  T  P  T  P  S  C  T  T  T  T  T  T  S  S

      610       620       630       640       650       660
       |         |         |         |         |         |
ACCCCCGGCGTCAAGGCCACGGCGGCGCAGAACATCGTCAAGCTGGTGCAGTCGTACGGC
 T  P  G  V  K  A  T  A  A  Q  N  I  V  K  L  V  Q  S  Y  G

      670       680       690       700       710       720
       |         |         |         |         |         |
GCGCGCATCGACGGCGTCGGCCTGCAGTCGCACTTCATCGTGGGCCAGACGCCCAGCACG
 A  R  I  D  G  V  G  L  Q  S  H  F  I  V  G  Q  T  P  S  T

      730       740       750       760       770       780
       |         |         |         |         |         |
AGCGCCCAGCAGCAGAACATGGCTGCCTTCACCGCGCTGGGCGTCGAGGTCGCCATCACC
 S  A  Q  Q  Q  N  M  A  A  F  T  A  L  G  V  E  V  A  I  T

      790       800       810       820       830       840
       |         |         |         |         |         |
GAGCTCGACATCCGCATGCAGCTGCCCGAGACGTCCGCGCAGCTGACACAGCAGGCGACC
 E  L  D  I  R  M  Q  L  P  E  T  S  A  Q  L  T  Q  Q  A  T
```

FIG. 11CONT'D

```
      850        860        870        880        890        900
       |          |          |          |          |          |
GACTACCAGAGCACGGTCCAGGCCTGCGTCAACACCGACAGCTGCGTCGGCATCACCCTC
D  Y  Q  S  T  V  Q  A  C  V  N  T  D  S  C  V  G  I  T  L

      910        920        930        940        950        960
       |          |          |          |          |          |
TGGGACTGGACCGACAAGTACTCGTGGGTGCCCAGCACCTTCTCAGGCTGGGGCGACGCC
W  D  W  T  D  K  Y  S  W  V  P  S  T  F  S  G  W  G  D  A

      970        980        990       1000       1010       1020
       |          |          |          |          |          |
TGTCCCTGGGACGACAACTACCAGAAGAAGCCCGCGTACAACGGCATCCTCACTGCTCTG
C  P  W  D  D  N  Y  Q  K  K  P  A  Y  N  G  I  L  T  A  L

     1030       1040       1050       1060       1070       1080
       |          |          |          |          |          |
GGAGGCACGCCCTCCTCCAGTACCAGCTACACCCTCACGCCGACGACGACCCCGAGCGGC
G  G  T  P  S  S  S  T  S  Y  T  L  T  P  T  T  T  P  S  G

     1090       1100       1110       1120       1130       1140
       |          |          |          |          |          |
GGCAGTGGCAGCCCGACTGACGTGGCTCAGCATTGGGAGCAGTGCGGTGGCCTGGGGCTG
G  S  G  S  P  T  D  V  A  Q  H  W  E  Q  C  G  G  L  G  L

     1150       1160       1170       1180       1190       1200
       |          |          |          |          |          |
GGACTGGGGCCGACGGTTTGCGCCAGTGGCTTCACTTGCACTGTCATCAACGAGTATTAC
G  L  G  P  T  V  C  A  S  G  F  T  C  T  V  I  N  E  Y  Y

     1210
       |
TCGCAGTGTCTGTAA
S  Q  C  L  -
```

FIG. 11 CONT'D

FIG. 12

gactcttagk kycccgtgca gcaggcaggg gacatccact cgagtccaat aatcattacc
tgacagctta gactrgacta attaattaaa attacccyta gtcagtcact gtcagaccat
tctaaggtta agtacgaaga cgaagccgga ttgccccgcg acacgaaaca agctacctat
tactctacgg agtactcccg cactggacgc agactaaaca gtccctgctt ccatgtcgga
atgtcggctt ttgttgtatg tagctagcta gctacctgca tacgttccag aacgatggat
gatggctgct ttcattgctt ttgcttttgc tttacctttt ttttttttt ttttgtgatt
gctgatcatg tgttccgaca gaagcttgta cagttccgat cgatctttct attcagccta
aaattaaatg gattgatttc atttggattg attgatggat taattgagtg agtgagtgcg
ccggacgaaa ggaacaattc attgcgttta ttgcattgca tcaccaagat tgtcttgtta
tatatacctg atcttcatct cccgtctcct ccaaatcctg gaaccaaatc tttctgaata
acagcagact tgcttgacga tcgatcgatc gattgattca agatggttcg cctcagtcca
gtcctgctgg catcgatcgc aggctctggc ctgcctctgt acgcacaagc agccggcctc
aacaccgccg ccaaagccgt cggcctgaaa tacttcggca cggcgaccga caaccccgaa
ctgagcgaca ccgcgtacga gacggagctg aacaacacgc aggatttcgg gcagttgaca
cctgcgaatt cgatgaaagt gagtctgaca cccccccccc cccctgggcg agtgagtgag
ttcgacgctg atggtttttg cagtgggacg caaccgagcc ccagcaaaac actttcacgt
tcagcggcgg cgatcagatc gctaacctgg ccaaggcgaa tggccagatg ttgaggtgcc
ataatcttgt ttggtataat cagttgccgt cgtggggtat gtatagtacc tgcgtgcttg
tttgtaatga ttgtcttggc tgatttgtga agtcaccggt ggatcctgga ccaacgagac
gctgcttgct gccatgaaga atcacatcac aaacgtcgtt acccattaca agggccagtg
ctatgcatgg gatgtcgtga atgagggtgc gtccatataa ttgctggtta ctatcgagag
gatcagctaa tgacgacagc cctcaacgac gacggcacct accgcagcaa cgtcttctac
cagtatatcg gggaggctac atcccccatc gccttcgcga cgccgccgcc gccgaccccg
acgccaagct gtactacaac gactacaaca tcgagtaccc ccggcgtcaa ggccacggcg
gcgcagaaca tcgtcaagct ggtgcagtcg tacggcgcgc gcatcgacgg cgtcggcctg
cagtcgcact tcatcgtggg ccagacgccc agcacgagcg cccagcagca gaacatggct
gccttcaccg cgctgggcgt cgaggtcgcc atcaccgagc tcgacatccg catgcagctg
cccgagacgt ccgcgcagct gacacagcag gcgaccgact accagagcac ggtccaggcc
tgcgtcaaca ccgacagctg cgtcggcatc accctctggg actggaccga caagtactcg
tgggtgccca gcaccttctc aggctggggc gacgcctgtc cctgggacga caactaccag
aagaagcccg cgtacaacgg catcctcact gctctgggag gcacgccctc ctccagtacc
agctacaccc tcacgccgac gacgaccccg agcggcggca gtggcagccc gactgacgtg
gctcagcatt gggagcagtg cggtggcctg gggctgggac tggggccgac ggtttgcgcc
agtggcttca cttgcactgt catcaacgag tattactcgc agtgtctgta atctgtcagt
aggatggtaa aagagagggg tgactgaagt ttcattgtac atacacatcc aaaaaaaaag
cgtgttgtat cggcgaaaat gctaagtcca attccmcgat tctattaatg ttcctgcgac
agggtattgc yccatgaatt ccaacaacag cccgmccaga agtaaaaggc tgagaaatga
agaccmaaga gcaggctctg aaaaccaatt agaatgtcga cgccagtatg ctcgcagtac
agtacagtcc aatgaagcaa gccccc

FIG. 13

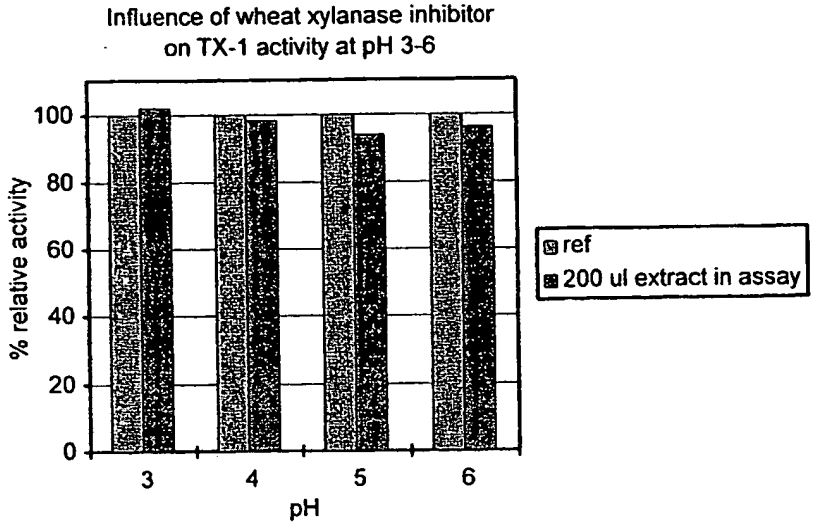
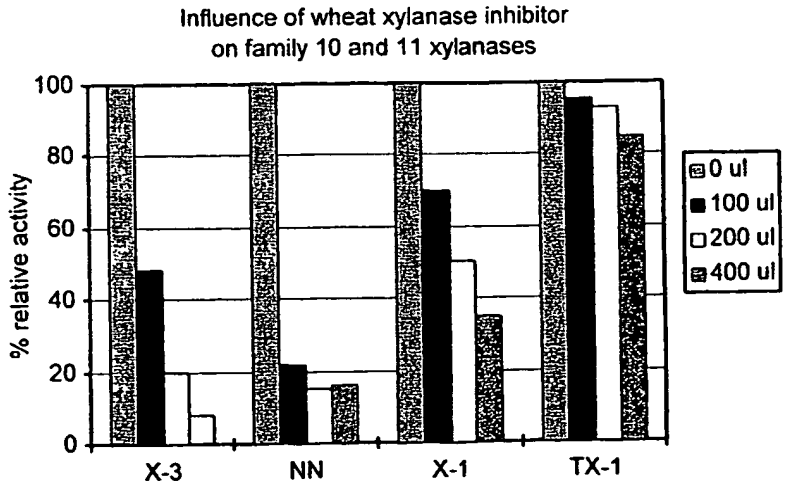


FIG. 14



Family 10 xylanases: TX-1 from *T.emersonii* and X-3: from *A. niger.*
Family 11 xylanases: X-1 from *A.niger* and NN (Biofeed Wheat) from Novo Nordisk

FIG. 15

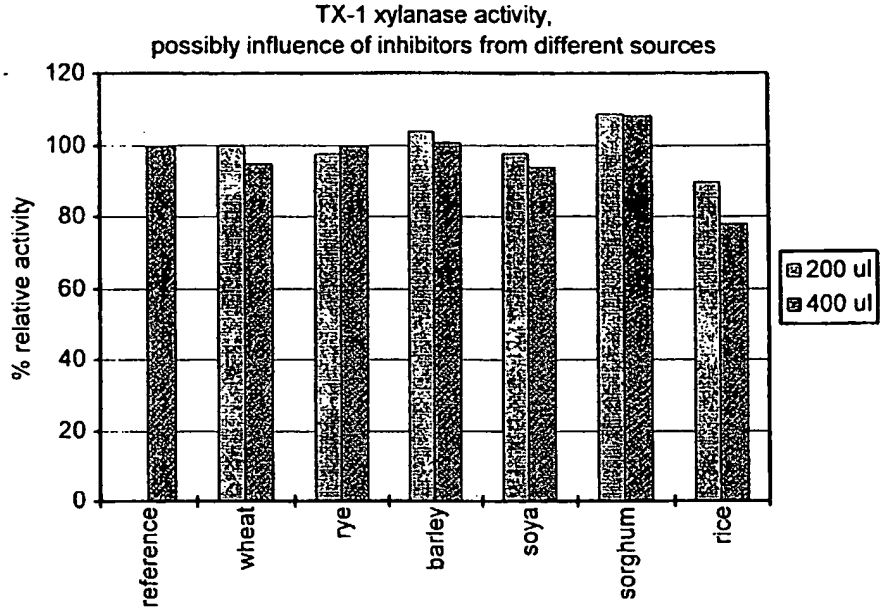
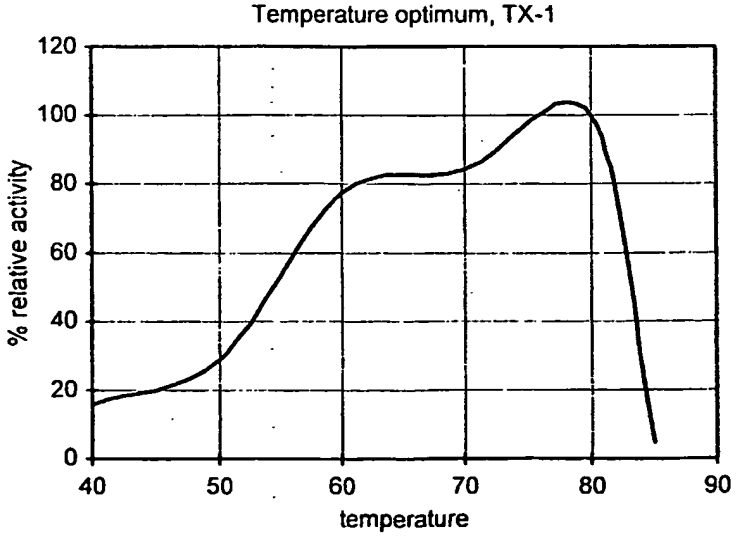

TX-1 xylanase activity,
possibly influence of inhibitors from different sources
% relative activity
120
100
80
60
40
20
0
reference
wheat
rye
barley
soya
sorghum
rice
200 ul
400 ul

FIG. 16


Temperature optimum, TX-1
% relative activity
120
100
80
60
40
20
0
40
50
60
70
80
90
temperature